IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

10X GENOMICS, INC. and PRESIDENT AND FELLOWS OF HARVARD COLLEGE, Plaintiffs, v. NANOSTRING TECHNOLOGIES, INC., Defendant.	C.A. No. 22-261 (MFK) **JURY TRIAL DEMANDED**
NANOSTRING TECHNOLOGIES, INC., Counterclaim-Plaintiff, v. 10X GENOMICS, INC., Counterclaim-Defendant.	

NANOSTRING TECHNOLOGIES, INC. FIRST AMENDED ANSWER, AFFIRMATIVE DEFENSES, AND COUNTERCLAIMS TO PLAINTIFFS' FIRST AMENDED COMPLAINT

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Date: July 29, 2022

Defendant NanoString Technologies, Inc. ("NanoString") hereby provides its Answer to the First Amended Complaint ("FAC") of 10x Genomics, Inc. ("10x") and President and Fellows of Harvard College ("Harvard") (collectively "Plaintiffs"), and amended Counterclaim as against 10x ("Counterclaim-Defendant") as set forth below. Unless expressly admitted, NanoString denies each and every allegation in Plaintiffs' FAC. To the extent the allegations in the FAC purport to characterize the nature or contents of the Exhibits to the FAC, NanoString lacks sufficient knowledge or information to form a belief as to the truth of those allegations and on that basis denies them. Additionally, to the extent that the headings or any other non-numbered statements in the FAC contain any allegations, NanoString denies each and every such allegation.

NATURE OF THE ACTION

1. NanoString admits that the FAC purports to state a claim for patent infringement of United States Patent Nos. 10,227,639 ("the 639 Patent), 11,021,737 ("the 737 Patent"), 11,293,051 ("the 051 Patent"), 11,293,052 ("the 052 Patent"), and 11,293,054 ("the 054 Patent") (collectively, the "Asserted Patents") arising under the patent laws of the United States, Title 35, United States Code, including 35 U.S.C. § 271. Except as so admitted, NanoString denies any remaining allegations in paragraph 1.

THE PARTIES

2. Admitted.

3. Denied.

4. NanoString lacks sufficient knowledge or information to form a belief as to the truth of the allegations set forth in paragraph 4 and on that basis denies them.

5. NanoString admits that it is a Delaware Corporation with its principal place of business is located in Seattle, WA. Except as so admitted, NanoString denies any remaining allegations in paragraph 5.

6. Denied.

JURISDICTION AND VENUE

7. NanoString incorporates by reference and restates its responses to paragraphs 1-6 of the FAC as though fully set forth herein.

8. NanoString admits that the FAC purports to state a claim for patent infringement arising under the patent laws of the United States, 35 U.S.C. §§ 1, et seq., including in particular 35 U.S.C. §§ 271. NanoString further admits that this Court has subject matter jurisdiction over causes of action for alleged patent infringement pursuant to 28 U.S.C. §§ 1331 and 1338(a).

9. NanoString admits that it is an entity organized under the laws of Delaware and that venue is proper in this District. Except as so admitted, NanoString denies any remaining allegations of paragraph 9.

A. Response to Allegations Regarding 10x's Products

10. Denied.

11. Denied.

12. Denied.

13. Denied.

14. Denied.

B. Response To Allegations Regarding 10x and *In Situ* Technologies

15. Paragraph 15 of the FAC includes allegations that are vague, ambiguous, and incomplete, and on that basis NanoString denies the allegations of paragraph 15.

16. NanoString lacks sufficient knowledge or information to form a belief as to the truth of the allegations set forth in paragraph 16 and on that basis denies them.

17. NanoString lacks sufficient knowledge or information to form a belief as to the truth of the allegations set forth in paragraph 17 and on that basis denies them.

C. Response to Allegations Regarding NanoString's Products

18. NanoString admits that it announced the launch of its Technology Access Program for the Spatial Molecular Imager Platform in March 2021. NanoString further admits that it issued a press release referencing the new CosMx Spatial Molecular Imager in November 2021. NanoString admits that Paragraph 18 accurately quotes a portion of NanoString's website. Paragraph 18 of the FAC further includes allegations that purport to characterize technical aspects of NanoString's products in a manner that is vague, ambiguous, and incomplete, and on that basis NanoString denies the remaining allegations of paragraph 18.

19. Denied.

20. NanoString admits that it markets certain products under the trade name CosMx. Paragraph 20 of the FAC further includes allegations that purport to characterize technical aspects of NanoString's products in a manner that is vague, ambiguous, and incomplete, and on that basis NanoString denies the remaining allegations of paragraph 20.

D. Response to Allegations Regarding the Patents In Suit

21. Denied.

22. NanoString admits that, on its face, the 639 Patent states that it was issued on March 12, 2019 and that it lists the named inventors as Daniel Levner, Jehyuk Lee, George M. Church, and Michael Super. Except as so admitted, NanoString denies any remaining allegations of paragraph 22.

23. NanoString lacks sufficient knowledge or information to form a belief as to the truth of the allegations set forth in paragraph 23 and on that basis denies them.

24. NanoString admits that, on its face, the 737 Patent states that it was issued on June 1, 2021 and that it lists the named inventors as Daniel Levner, Jehyuk Lee, George M. Church, and Michael Super. Except as so admitted, NanoString denies any remaining allegations of paragraph 24.

25. NanoString lacks sufficient knowledge or information to form a belief as to the truth of the allegations set forth in paragraph 25 and on that basis denies them.

26. NanoString admits that, on its face, the 051 Patent states that it was issued on April 5, 2022, and that it lists the named inventors as Daniel Levner, Jehyuk Lee, George M. Church, and Michael Super. Except as so admitted, NanoString denies any remaining allegations of paragraph 26.

27. NanoString lacks sufficient knowledge or information to form a belief as to the truth of the allegations set forth in paragraph 27 and on that basis denies them.

28. NanoString admits that, on its face, the 052 Patent states that it was issued on April 5, 2022, and that it lists the named inventors as Daniel Levner, Jehyuk Lee, George M. Church, and Michael Super. Except as so admitted, NanoString denies any remaining allegations of paragraph 28.

29. NanoString lacks sufficient knowledge or information to form a belief as to the truth of the allegations set forth in paragraph 29 and on that basis denies them.

30. NanoString admits that, on its face, the 054 Patent states that it was issued on April 5, 2022, and that it lists the named inventors as Daniel Levner, Jehyuk Lee, George M. Church,

and Michael Super. Except as so admitted, NanoString denies any remaining allegations of paragraph 30.

31. NanoString lacks sufficient knowledge or information to form a belief as to the truth of the allegations set forth in paragraph 31 and on that basis denies them.

COUNT I

32. NanoString incorporates and restates by reference its responses to paragraphs 1-31 of the FAC as though fully set forth herein.

33. Denied.

34. Denied.

35. Denied.

COUNT II

36. NanoString incorporates and restates by reference its responses to paragraphs 1-35 of the FAC as though fully set forth herein.

37. Denied.

38. Denied.

39. Denied.

COUNT III

40. NanoString incorporates and restates by reference its responses to paragraphs 1-39 of the FAC as though fully set forth herein.

41. Denied.

42. Denied.

43. Denied.

COUNT IV

44. NanoString incorporates and restates by reference its responses to paragraphs 1-43 of the FAC as though fully set forth herein.

45. Denied.

46. Denied.

47. Denied.

COUNT V

48. NanoString incorporates and restates by reference its responses to paragraphs 1-47 of the FAC as though fully set forth herein.

49. Denied.

50. Denied.

51. Denied.

PRAYER FOR RELIEF

NanoString denies that Plaintiffs are entitled to any relief whatsoever, including the relief stated in paragraphs A through F, from either NanoString or the Court. Plaintiffs' prayer for relief should be denied in its entirety.

AFFIRMATIVE DEFENSES

NanoString hereby sets forth defenses to the FAC in order to place Plaintiffs on notice regarding applicable defenses. By listing any matter as a defense herein, NanoString does not assume the burden of proving any matter upon which Plaintiffs, or any other party, bears the burden of proof under applicable law.

FIRST DEFENSE – NON-INFRINGEMENT

NanoString has not infringed, and is not infringing directly, indirectly, contributorily, by inducement, or in any other manner any valid and enforceable claim of the Asserted Patents, either literally or under the doctrine of equivalents.

SECOND DEFENSE – INVALIDITY

The asserted claims of the Asserted Patents are invalid for failing to comply with one or more of the requirements for patentability under, including, but not limited to 35 U.S.C. §§ 101, 102, 103, 112 et seq., and the rules, regulations, and laws pertaining to those provisions, including the applicable provisions of Title 37 of the Code of Federal Regulations.

THIRD DEFENSE – 35 U.S.C. § 287

Plaintiffs' patent infringement claims and Prayer for Relief are limited by 35 U.S.C. § 287.

FOURTH DEFENSE – ADEQUATE REMEDY AT LAW

Plaintiffs have an adequate remedy at law and the alleged injury to Plaintiffs is not immediate or irreparable. Accordingly, Plaintiffs are not entitled to injunctive relief even if it were able to establish liability.

FIFTH DEFENSE – NO EXCEPTIONAL CASE

NanoString has not engaged in any conduct that would make this an exceptional case or that would entitle Plaintiffs to an award of attorneys' fees.

SIXTH DEFENSE – FAILURE TO STATE A CLAIM

Plaintiffs' FAC fails to state a claim upon which relief may be granted.

NANOSTRING'S AMENDED COUNTERCLAIMS AGAINST PLAINTIFFS

In further response to Plaintiffs' FAC, Defendant and Counter-Plaintiff NanoString hereby asserts these Counterclaims against Plaintiff Harvard and Counter-Defendant 10x and alleges as follows.

NATURE OF THE ACTION

1. In response to Plaintiffs' allegations in the FAC, NanoString seeks a declaratory judgment that it has not infringed the Asserted Patents and that the Asserted Patents are invalid, arising under the Patent Laws of the United States 35 U.S.C. § 271 *et seq.* NanoString also seeks a finding of infringement of U.S. Patent No. 11,377,689 ("the '689 Patent") by Counter-Defendant 10x.

THE PARTIES

2. Plaintiff 10x is a Delaware corporation with its principal place of business at 6230 Stoneridge Mall Road, Pleasanton, CA 94588.

3. Plaintiff Harvard is a Massachusetts educational institution according to its allegations in the FAC.

4. NanoString is a Delaware corporation with its principal place of business at 530 Fairview Ave. N, Seattle, WA 98109.

JURISDICTION AND VENUE

5. These Counterclaims arise under the Patent Act, and this Court has subject matter jurisdiction pursuant to 28 U.S.C. §§ 1331 & 1338, the Declaratory Judgement Act, 28 U.S.C. §§ 2201 & 2202, and the Patent Laws of the United States, 35 U.S.C. § 1, *et seq*

6. The Court has personal jurisdiction over 10x and Harvard because 10x is a Delaware corporation and both 10x and Harvard have consented to jurisdiction in this District by filing their FAC in this action.

7. Venue is proper in this Court pursuant to 28 U.S.C. §§ 1391 and 1400(b) because 10x is a Delaware corporation and both 10x and Harvard have consented to this venue by filing their original complaint in this action.

BACKGROUND

8. NanoString is the pioneer in the field of spatial biology. As a respected, leading innovator of translational tools, NanoString has developed and brought to market breakthrough technologies that enable scientists across the globe to envision molecular interactions in three dimensions. NanoString's GeoMx Digital Spatial Profiler ("GeoMx DSP") product is the first of its kind – it uniquely combines high-plex and high-throughput spatial profiling, which allows researchers to rapidly and quantitatively assess the biological implications of heterogeneity within tissues. Since its launch, GeoMx DSP has been amply described in prestigious scientific publications, and studies performed using GeoMx DSP have been widely presented at industry conferences.

9. On July 5, 2022, the United States Patent and Trademark Office duly and legally issued the United States Patent No. 11,377,689 ("the '689 Patent"), entitled "Chemical Compositions and Uses Thereof". The named inventors of the '689 Patent are Joseph Beechem, Dae Kim, Margaret Hoang, Mark Gregory, Erin Piazza, and Denise Zhou. By operation of law and as a result of written assignment agreements, Counterclaim-Plaintiff NanoString obtained the entire right, title, and interest to and in the '689 Patent. The '689 Patent is attached hereto. Ex. 1.

10. Since its issuance, NanoString has owned the '689 Patent.

11. As its Abstract explains, the '689 Patent is directed to an improved and novel way of spatially detecting target analytes in regions of interest. More specifically, the method claimed in the '689 Patent permits the simultaneous, multiplexed detection and quantification of protein

and/or nucleic acid expression in a user-defined region of a tissue, cell, and/or subcellular structure within a cell, while maintaining the morphological context of the sample.

12. The claimed invention of the '689 Patent is an improvement over the prior art means of detecting target analytes in a sample. Conventional immunohistochemical methods allow for simultaneous detection of six to ten protein targets at most. Similarly, *in situ* hybridization methods are limited to less than ten nucleic acid targets. '689 Patent at 16:30-32. While useful, these methods do not enable the simultaneous detection of a large number of genes, proteins, or other biologically active molecules in the same sample. Laser microdissection is able to capture many genes, but it is limited to a small number of locations and very expensive. The claimed invention of the '689 Patent uniquely addresses and solves these problems. As such, the invention offers a spatial profiling method for RNA and/or protein in a tissue sample of much higher plex and sensitivity, providing researchers the ability to look at many biological targets at many locations in the same sample at the same time. As the '689 Patent explains, the "present disclosure provides detection of large combinations of nucleic acid targets and/or protein targets from a defined region of a sample. The present disclosure provides an increase in objective measurements by digital quantification and increased reliability and consistency, thereby enabling comparison of results among multiple centers." *Id.* at 16:37-43. Specifically, more than 1000 targets can be detected, and "[t]here is no pre-defined upper limit to the number of regions of interest and comparisons that can be made." *Id.* at 16:21-23; 40:61-64. Furthermore, the invention is compatible with existing, readily available sequencing technologies, making it accessible to a large number of users; it also allows for an economical assay design, as inexpensive and widely-available synthetic DNA oligonucleotides can be used instead of more expensive probes. *Id.* at Abstract; 37:50-55.

13. On information and belief, 10x launched and began shipping its commercial spatial profiling products, Visium Spatial system ("Visium") and related products in November, 2019. *See* https://www.globenewswire.com/news-release/2019/11/26/1952684/0/en/10x-Genomics-Begins-Shipments-of-Visium-Spatial-Gene-Expression-Solution.html.

14. Visium and related products are all products, components, and services that are made, used, performed, offered to sell, sold, and/or imported in the United States by or on behalf of 10x in connection with 10x's Visium. Visium and related products include, for example and without limitation, Visium CytAssist, Visium Spatial Gene Expression slides, Visium Spatial Gene Expression reagents, and analysis and visualization software, Space Ranger and Loupe Browser, and Certified Service Providers (CSP), that when used together allow researchers to "map the whole transcriptome within the tissue context" and "[c]ombine histological and gene expression data with easy-to-use software." *See* https://www.10xgenomics.com/products/spatial-gene-expression.

15. Visium and related products provide spatial profiling for protein and/or RNA in a tissue sample. 10x offers benchtop instrument, slides, reagents, and software for protein and RNA analysis using workflows that are compatible with standard next generation sequencing (NGS) applications. 10x markets Visium as designed to "discover and reveal the spatial organization of cell types, states, and biomarkers." *See* https://pages.10xgenomics.com/rs/446-PBO-704/images/10x_LIT059_ProductSheet_VisiumSpatialGeneExpression_Letter_digital.pdf. Furthermore, 10x states that Visium is able to "[s]patially profile RNA expression for over 18,000 genes in human and mouse FFPE samples with high resolution across entire tissue sections," *see* https://pages.10xgenomics.com/rs/446-PBO-704/images/10x_LIT000128_PS_Spatial_biology_without_limits_Spatial_gene_expression_in_F

FPE.pdf, offers "tissue profiling with transcriptomics and protein co-detection," *see* https://www.10xgenomics.com/products/spatial-proteogenomics, allows users to "define regions of interest," *see* https://pages.10xgenomics.com/rs/446-PBO-704/images/10x_LIT088_RevA_ProductSheet_Immunofluorescence%20Capability_Letter_digital.pdf, and is "[e]asy to integrate with current histological laboratory methods and tools for tissue analysis." *Id.*

16. 10x practices the '689 Patent by using Visium and related products on behalf of its own scientists, researchers, and its Visium customers for various purposes, including without limitation research, development, sales, and support.

17. 10x infringes the '689 Patent by providing customers with Certified Service Providers (CSP), who offer support for complete end-to-end Visium workflows and perform various tasks, from sample preparation to library generation to data processing, for its customers. *See* https://www.10xgenomics.com/service-providers?query=&page=1.

18. 10x additionally infringes the '689 Patent by making, using, selling, offering for sale, importing into the United States, and supplying from the United States the patented inventions and/or its components, including without limitation Visium, Visium CytAssist, Visium Spatial Gene Expression slides, Visium Spatial Gene Expression reagents, and analysis and visualization software, Space Ranger and Loupe Browser, and CSP.

19. Through the development and subsequent making, using, selling, offering for sale, importing and exporting of its Visium and related products, and performing every step of the patented invention by using products, services, devices, systems, and/or components of the systems that embody the patented inventions, such as Visium and related products, 10x has and

continues to directly infringe, contributorily infringe, and/or induce the infringement of the '689 Patent.

FIRST COUNT

(Declaration of Non-Infringement of United States Patent No. 10,227,639)

20. NanoString incorporates by references and restates the preceding Paragraphs 1-18 of its Counterclaims as though fully set forth herein.

21. Plaintiffs have brought an action asserting the 639 Patent against NanoString.

22. Plaintiff Harvard has alleged that it is the legal owner by assignment of the 639 Patent.

23. Plaintiff 10x has alleged that it is the exclusive licensee of the 639 Patent.

24. Plaintiffs have alleged and continue to allege that NanoString has infringed and continues to infringe one or more claims of the 639 Patent.

25. An actual controversy, within the meaning of 28 U.S.C. §§ 2201 and 2202, has arisen and exists between Plaintiffs and NanoString concerning whether NanoString has infringed and is infringing any valid and enforceable claim of the 639 Patent.

26. NanoString's products are not infringing directly or in any other manner any valid and enforceable claim of the 639 Patent.

27. For example, as described therein, when properly construed, the 639 Patent claims require analytes to be immobilized in the sample for analyte identification. In contrast, NanoString's CosMx SMI does not immobilize analytes for analyte identification.

28. Also, as described therein, when properly construed, the 639 Patent claims require pre-determined subsequences to form an identifier of analyte. In contrast, to the extent they are used in CosMx SMI, pre-determined subsequences are not identifiers, but intermediate sequences for hybridization of probes.

29. Furthermore, as described therein, when properly construed, the 639 Patent claims require a first decoder probe to be removed before the hybridization of the second decoder probe. In contrast, CosMx SMI do not remove any such decoder probes during hybridization.

30. Moreover, as described therein, when properly construed, the 639 Patent claims require identification of a probe for analyte identification. In contrast, NanoString's CosMx SMI does not identify a probe to identify an analyte.

31. Besides, as described therein, when properly construed, the 639 Patent claims require the use of two detectable labels for analyte identification. To the extent any accused products do not use two detectable labels for analyte identification, they do not infringe the 639 Patent.

32. By virtue of the foregoing, NanoString desires a judicial determination of its rights and duties with respect to any alleged infringement of the 639 Patent.

33. A judicial declaration is necessary and appropriate at this time so that the parties may proceed in accordance with their respective rights and duties determined by the Court.

SECOND COUNT

(Declaration of Non-Infringement of United States Patent No. 11,021,737)

34. NanoString incorporates by references and restates the preceding Paragraphs 1-32 of its Counterclaims as though fully set forth herein.

35. Plaintiffs have brought an action asserting the 737 Patent against NanoString.

36. Plaintiff Harvard has alleged that it is the legal owner by assignment of the 737 Patent.

37. Plaintiff 10x has alleged that it is the exclusive licensee of the 737 Patent.

38. Plaintiffs have alleged and continue to allege that NanoString has infringed and continues to infringe one or more claims of the 737 Patent.

39. An actual controversy, within the meaning of 28 U.S.C. §§ 2201 and 2202, has arisen and exists between Plaintiffs and NanoString concerning whether NanoString has infringed and is infringing any valid and enforceable claim of the 737 Patent.

40. NanoString's products are not infringing directly or in any other manner any valid and enforceable claim of the 737 Patent.

41. For example, as described therein, when properly construed, the 737 Patent claims require the analytes to be in the cell or tissue sample during analyte identification. In contrast, NanoString's CosMx SMI does not identify analytes in the cell or tissue sample.

42. Furthermore, as described therein, when properly construed, the 737 Patent claims require signal signatures to be associated with one or more pre-determined subsequences. In contrast, to the extent they are used in CosMx SMI, signal signatures are not associated with pre-determined subsequences, but with the hybridized decoder probes.

43. Moreover, as described therein, when properly construed, the 737 Patent claims require first decoder probe to be removed before the hybridization of the second decoder probe. In contrast, CosMx SMI does not remove the decoder probes during serial hybridization.

44. Besides, as described therein, when properly construed, the 737 Patent require a temporal order of signal signatures for analyte identification. To the extent any accused products do not use a temporal order of signal signatures for analyte identification, they do not infringe the 639 Patent.

45. By virtue of the foregoing, NanoString desires a judicial determination of its rights and duties with respect to any alleged infringement of the 737 Patent.

46. A judicial declaration is necessary and appropriate at this time so that the parties may proceed in accordance with their respective rights and duties determined by the Court.

THIRD COUNT

(Declaration of Non-Infringement of United States Patent No. 11,293,051)

47. NanoString incorporates by references and restates the preceding Paragraphs 1-45 of its Counterclaims as though fully set forth herein.

48. Plaintiffs have brought an action asserting the 051 Patent against NanoString.

49. Plaintiff Harvard has alleged that it is the legal owner by assignment of the 051 Patent.

50. Plaintiff 10x has alleged that it is the exclusive licensee of the 051 Patent.

51. Plaintiffs have alleged and continue to allege that NanoString has infringed and continues to infringe one or more claims of the 051 Patent.

52. An actual controversy, within the meaning of 28 U.S.C. §§ 2201 and 2202, has arisen and exists between Plaintiffs and NanoString concerning whether NanoString has infringed and is infringing any valid and enforceable claim of the 051 Patent.

53. NanoString's products are not infringing directly or in any other manner any valid and enforceable claim of the 051 Patent.

54. For example, as described therein, when properly construed, the 051 Patent claims require the analytes to be in the cell or tissue sample for analyte identification. In contrast, NanoString's CosMx SMI does not identify analytes in the cell or tissue sample.

55. Also, as described therein, when properly construed, the 051 Patent claims require a temporal order of signal signatures to correspond to a location in a cell or tissue sample. In contrast, NanoString's CosMx SMI does not use a temporal order of signal signatures corresponding to a location.

56. Furthermore, as described therein, when properly construed, the 051 Patent claims require first plurality of signal signatures to be removed from the cell or tissue sample before the

second readout cycle. In contrast, CosMx SMI does not remove the first signal signatures from the cell or tissue sample, but from the bound probes.

57. Moreover, as described therein, when properly construed, the 051 Patent claims require the first subset of detection reagents associated with the first set of decoding reagents and the second set of detection reagents associated with the second set of decoding reagents to overlap. To the extent any of the accused products do not let the first subset of detection reagents and the second subset of detection reagents to overlap, they do not infringe the 051 Patent.

58. By virtue of the foregoing, NanoString desires a judicial determination of its rights and duties with respect to any alleged infringement of the 051 Patent.

59. A judicial declaration is necessary and appropriate at this time so that the parties may proceed in accordance with their respective rights and duties determined by the Court.

FOURTH COUNT

(Declaration of Non-Infringement of United States Patent No. 11,293,052)

60. NanoString incorporates by references and restates the preceding Paragraphs 1-58 of its Counterclaims as though fully set forth herein.

61. Plaintiffs have brought an action asserting the 052 Patent against NanoString.

62. Plaintiff Harvard has alleged that it is the legal owner by assignment of the 052 Patent.

63. Plaintiff 10x has alleged that it is the exclusive licensee of the 052 Patent.

64. Plaintiffs have alleged and continue to allege that NanoString has infringed and continues to infringe one or more claims of the 052 Patent.

65. An actual controversy, within the meaning of 28 U.S.C. §§ 2201 and 2202, has arisen and exists between Plaintiffs and NanoString concerning whether NanoString has infringed and is infringing any valid and enforceable claim of the 052 Patent.

66. NanoString's products are not infringing directly or in any other manner any valid and enforceable claim of the 052 Patent.

67. For example, as described therein, when properly construed, the 052 Patent claims require the analyte to be at a location in a biological sample during identification. In contrast, NanoString's CosMx SMI does not identify the analyte at the location in a biological sample.

68. Furthermore, as described therein, when properly construed, the 052 Patent claims require the first optical signal to be removed from the location in the biological sample before the second readout cycle. In contrast, CosMx SMI does not remove the first optical signal from the sample, but from the bound probes.

69. Moreover, as described therein, when properly construed, the 052 Patent claims require the detection of multiple signal signatures and the absence thereof during analyte identification. To the extent any of the accused products do not detect multiple signal signatures for analyte detection, they do not infringe the 052 Patent.

70. By virtue of the foregoing, NanoString desires a judicial determination of its rights and duties with respect to any alleged infringement of the 052 Patent.

71. A judicial declaration is necessary and appropriate at this time so that the parties may proceed in accordance with their respective rights and duties determined by the Court.

FIFTH COUNT

(Declaration of Non-Infringement of United States Patent No. 11,293,054)

72. NanoString incorporates by references and restates the preceding Paragraphs 1-70 of its Counterclaims as though fully set forth herein.

73. Plaintiffs have brought an action asserting the 054 Patent against NanoString.

74. Plaintiff Harvard has alleged that it is the legal owner by assignment of the 054 Patent.

75. Plaintiff 10x has alleged that it is the exclusive licensee of the 054 Patent.

76. Plaintiffs have alleged and continue to allege that NanoString has infringed and continues to infringe one or more claims of the 054 Patent.

77. An actual controversy, within the meaning of 28 U.S.C. §§ 2201 and 2202, has arisen and exists between Plaintiffs and NanoString concerning whether NanoString has infringed and is infringing any valid and enforceable claim of the 054 Patent.

78. NanoString's products are not infringing directly or in any other manner any valid and enforceable claim of the 054 Patent.

79. For example, as described therein, when properly construed, the 054 Patent claims require generation of signal signatures in a cell or tissue sample. In contrast, NanoString's CosMx SMI does not generate a signal signatures in a cell or tissue sample.

80. Furthermore, as described therein, when properly construed, the 054 Patent claims require a nucleic acid label coupled to a probe to permit the said probe to bind to an analyte. In contrast, to the extent it is used in NanoString's CosMx SMI, the nucleic acid label does not permit the probe to bind to an analyte. Rather, the probe is itself designed to target an analyte.

81. Moreover, as described therein, when properly construed, the 054 Patent claims require first decoder probe to be removed before the hybridization of the second decoder probe. In contrast, CosMx SMI do not remove the decoder probes during serial hybridization.

82. By virtue of the foregoing, NanoString desires a judicial determination of its rights and duties with respect to any alleged infringement of the 054 Patent.

83. A judicial declaration is necessary and appropriate at this time so that the parties may proceed in accordance with their respective rights and duties determined by the Court.

SIXTH COUNT

(Declaration of Invalidity of U.S. Patent No. 10,227,639)

84. NanoString incorporates by references and restates the preceding Paragraphs 1-82 of its Counterclaims as though fully set forth herein.

85. Plaintiffs have brought an action asserting the 639 Patent against NanoString.

86. Plaintiff Harvard has alleged that it is the legal owner by assignment of the 639 Patent.

87. Plaintiff 10x has alleged that it is the exclusive license of the 639 Patent.

88. Plaintiffs have alleged and continues to allege that NanoString has infringed and continues to infringe one or more claims of the 639 Patent.

89. An actual controversy, within the meaning of 28 U.S.C. §§ 2201 and 2202, has arisen and exists between Plaintiffs and NanoString concerning whether NanoString has infringed and is infringing any valid and enforceable claim of the 639 Patent.

90. The claims of the 639 Patent are invalid for failing to comply with the provisions of the Patent Laws, Title 35 of the United States Code, including without limitation one or more of 35 U.S.C. §§ 101, 102, 103, 112, and/or 116, and/or the rules, regulations and law pertaining thereto.

91. For example, the asserted claims of the Asserted Patents are invalid under 35 U.S.C. §§ 102 and/or 103 at least in view of U.S. Patent No. 10,961,566 ("Chee"), alone or in combination with additional prior art, including U.S. Patent App. Pub. No. 2005/0064435 ("Su"), Göransson et al., A single molecule array for digital targeted molecular analyses, 37 Nucleic Acids Research e7 (2008) ("Göransson"), or U.S. Patent No. 8,741,566 ("Winther"), which disclose and/or render obvious all elements of the claims of the Asserted Patents.

92. All claims of the Asserted Patents are further invalid for failure to satisfy the requirements of 35 U.S.C. § 112. For example, the claim term "temporal order", read in light of

the specification and the prosecution history, fails to inform, with reasonable certainty, those skilled in the art of the boundaries of protected subject matter, and therefore does not meet the definiteness standard.

93. Moreover, the specifications of the Asserted Patents fail to contain a written description of the claims or sufficient information to enable a person of ordinary skill in the art to practice the full scope of the claims. For example, there is a lack of an adequate written description and a lack of enablement for signal detection in the cell or tissue sample.

94. By virtue of the foregoing, NanoString desires a judicial determination of its rights and duties with respect to any alleged infringement of the 639 Patent.

95. A judicial declaration is necessary and appropriate at this time so that the parties may proceed in accordance with their respective rights and duties determined by the Court.

SEVENTH COUNT

(Declaration of Invalidity of U.S. Patent No. 11,021,737)

96. NanoString incorporates by references and restates the preceding Paragraphs 1-94 of its Counterclaims as though fully set forth herein.

97. Plaintiffs have brought an action asserting the 737 Patent against NanoString.

98. Plaintiff Harvard has alleged that it is the legal owner by assignment of the 737 Patent.

99. Plaintiff 10x has alleged that it is the exclusive license of the 737 Patent.

100. Plaintiffs have alleged and continues to allege that NanoString has infringed and continues to infringe one or more claims of the 737 Patent.

101. An actual controversy, within the meaning of 28 U.S.C. §§ 2201 and 2202, has arisen and exists between Plaintiffs and NanoString concerning whether NanoString has infringed and is infringing any valid and enforceable claim of the 737 Patent.

102. The claims of the 737 Patent are invalid for failing to comply with the provisions of the Patent Laws, Title 35 of the United States Code, including without limitation one or more of 35 U.S.C. §§ 101, 102, 103, 112, and/or 116, and/or the rules, regulations and law pertaining thereto.

103. For example, the asserted claims of the Asserted Patents are invalid under 35 U.S.C. §§ 102 and/or 103 at least in view of U.S. Patent No. 10,961,566 ("Chee"), alone or in combination with additional prior art, including U.S. Patent App. Pub. No. 2005/0064435 ("Su"), Göransson et al., A single molecule array for digital targeted molecular analyses, 37 Nucleic Acids Research e7 (2008) ("Göransson"), or U.S. Patent No. 8,741,566 ("Winther"), which disclose and/or render obvious all elements of the claims of the Asserted Patents.

104. All claims of the Asserted Patents are further invalid for failure to satisfy the requirements of 35 U.S.C. § 112. For example, the claim term "temporal order", read in light of the specification and the prosecution history, fails to inform, with reasonable certainty, those skilled in the art of the boundaries of protected subject matter, and therefore does not meet the definiteness standard.

105. Moreover, the specifications of the Asserted Patents fail to contain a written description of the claims or sufficient information to enable a person of ordinary skill in the art to practice the full scope of the claims. For example, there is a lack of an adequate written description and a lack of enablement for generating a three dimensional matrix of nucleic acids in situ in a cell or tissue sample and amplifying, detecting, and sequencing such nucleic acids within the matrix; there is also a lack of an adequate written description and a lack of enablement for analyte detection while allowing spatial movement of an analyte in a sample.

106. By virtue of the foregoing, NanoString desires a judicial determination of its rights and duties with respect to any alleged infringement of the 737 Patent.

107. A judicial declaration is necessary and appropriate at this time so that the parties may proceed in accordance with their respective rights and duties determined by the Court.

EIGHTH COUNT

(Declaration of Invalidity of U.S. Patent No. 11,293,051)

108. NanoString incorporates by references and restates the preceding Paragraphs 1-106 of its Counterclaims as though fully set forth herein.

109. Plaintiffs have brought an action asserting the 051 Patent against NanoString.

110. Plaintiff Harvard has alleged that it is the legal owner by assignment of the 051 Patent.

111. Plaintiff 10x has alleged that it is the exclusive license of the 051 Patent.

112. Plaintiffs have alleged and continues to allege that NanoString has infringed and continues to infringe one or more claims of the 051 Patent.

113. An actual controversy, within the meaning of 28 U.S.C. §§ 2201 and 2202, has arisen and exists between Plaintiffs and NanoString concerning whether NanoString has infringed and is infringing any valid and enforceable claim of the 051 Patent.

114. The claims of the 051 Patent are invalid for failing to comply with the provisions of the Patent Laws, Title 35 of the United States Code, including without limitation one or more of 35 U.S.C. §§ 101, 102, 103, 112, and/or 116, and/or the rules, regulations and law pertaining thereto.

115. For example, the asserted claims of the Asserted Patents are invalid under 35 U.S.C. §§ 102 and/or 103 at least in view of U.S. Patent No. 10,961,566 ("Chee"), alone or in combination with additional prior art, including U.S. Patent App. Pub. No. 2005/0064435 ("Su"), Göransson et

al., A single molecule array for digital targeted molecular analyses, 37 Nucleic Acids Research e7 (2008) ("Göransson"), or U.S. Patent No. 8,741,566 ("Winther"), which disclose and/or render obvious all elements of the claims of the Asserted Patents.

116. Moreover, the specifications of the Asserted Patents fail to contain a written description of the claims or sufficient information to enable a person of ordinary skill in the art to practice the full scope of the claims. For example, there is a lack of an adequate written description and a lack of enablement for generating a three dimensional matrix of nucleic acids in situ in a cell or tissue sample and amplifying, detecting, and sequencing such nucleic acids within the matrix; there is also a lack of an adequate written description and a lack of enablement for analyte detection while allowing spatial movement of an analyte in a sample.

117. By virtue of the foregoing, NanoString desires a judicial determination of its rights and duties with respect to any alleged infringement of the 051 Patent.

118. A judicial declaration is necessary and appropriate at this time so that the parties may proceed in accordance with their respective rights and duties determined by the Court.

NINTH COUNT

(Declaration of Invalidity of U.S. Patent No. 11,293,052)

119. NanoString incorporates by references and restates the preceding Paragraphs 1-117 of its Counterclaims as though fully set forth herein.

120. Plaintiffs have brought an action asserting the 052 Patent against NanoString.

121. Plaintiff Harvard has alleged that it is the legal owner by assignment of the 052 Patent.

122. Plaintiff 10x has alleged that it is the exclusive license of the 052 Patent.

123. Plaintiffs have alleged and continues to allege that NanoString has infringed and continues to infringe one or more claims of the 052 Patent.

124. An actual controversy, within the meaning of 28 U.S.C. §§ 2201 and 2202, has arisen and exists between Plaintiffs and NanoString concerning whether NanoString has infringed and is infringing any valid and enforceable claim of the 052 Patent.

125. The claims of the 052 Patent are invalid for failing to comply with the provisions of the Patent Laws, Title 35 of the United States Code, including without limitation one or more of 35 U.S.C. §§ 101, 102, 103, 112, and/or 116, and/or the rules, regulations and law pertaining thereto.

126. For example, the asserted claims of the Asserted Patents are invalid under 35 U.S.C. §§ 102 and/or 103 at least in view of U.S. Patent No. 10,961,566 ("Chee"), alone or in combination with additional prior art, including U.S. Patent App. Pub. No. 2005/0064435 ("Su"), Göransson et al., A single molecule array for digital targeted molecular analyses, 37 Nucleic Acids Research e7 (2008) ("Göransson"), or U.S. Patent No. 8,741,566 ("Winther"), which disclose and/or render obvious all elements of the claims of the Asserted Patents.

127. All claims of the Asserted Patents are further invalid for failure to satisfy the requirements of 35 U.S.C. § 112. For example, the claim terms "signal signature" and "temporal order", read in light of the specification and the prosecution history, fails to inform, with reasonable certainty, those skilled in the art of the boundaries of protected subject matter, and therefore does not meet the definiteness standard.

128. Moreover, the specifications of the Asserted Patents fail to contain a written description of the claims or sufficient information to enable a person of ordinary skill in the art to practice the full scope of the claims. For example, there is a lack of an adequate written description and a lack of enablement for analyte identification at a location in a biological sample.

129. By virtue of the foregoing, NanoString desires a judicial determination of its rights and duties with respect to any alleged infringement of the 052 Patent.

130. A judicial declaration is necessary and appropriate at this time so that the parties may proceed in accordance with their respective rights and duties determined by the Court.

TENTH COUNT

(Declaration of Invalidity of U.S. Patent No. 11,293,054)

131. NanoString incorporates by references and restates the preceding Paragraphs 1-129 of its Counterclaims as though fully set forth herein.

132. Plaintiffs have brought an action asserting the 054 Patent against NanoString.

133. Plaintiff Harvard has alleged that it is the legal owner by assignment of the 054 Patent.

134. Plaintiff 10x has alleged that it is the exclusive license of the 054 Patent.

135. Plaintiffs have alleged and continues to allege that NanoString has infringed and continues to infringe one or more claims of the 054 Patent.

136. An actual controversy, within the meaning of 28 U.S.C. §§ 2201 and 2202, has arisen and exists between Plaintiffs and NanoString concerning whether NanoString has infringed and is infringing any valid and enforceable claim of the 054 Patent.

137. The claims of the 054 Patent are invalid for failing to comply with the provisions of the Patent Laws, Title 35 of the United States Code, including without limitation one or more of 35 U.S.C. §§ 101, 102, 103, 112, and/or 116, and/or the rules, regulations and law pertaining thereto.

138. For example, the asserted claims of the Asserted Patents are invalid under 35 U.S.C. §§ 102 and/or 103 at least in view of U.S. Patent No. 10,961,566 ("Chee"), alone or in combination with additional prior art, including U.S. Patent App. Pub. No. 2005/0064435 ("Su"), Göransson et

al., A single molecule array for digital targeted molecular analyses, 37 Nucleic Acids Research e7 (2008) ("Göransson"), or U.S. Patent No. 8,741,566 ("Winther"), which disclose and/or render obvious all elements of the claims of the Asserted Patents.

139. All claims of the Asserted Patents are further invalid for failure to satisfy the requirements of 35 U.S.C. § 112. For example, the claim term "temporal order", read in light of the specification and the prosecution history, fails to inform, with reasonable certainty, those skilled in the art of the boundaries of protected subject matter, and therefore does not meet the definiteness standard.

140. Moreover, the specifications of the Asserted Patents fail to contain a written description of the claims or sufficient information to enable a person of ordinary skill in the art to practice the full scope of the claims. For example, there is a lack of an adequate written description and a lack of enablement for signal detection in the cell or tissue sample.

141. By virtue of the foregoing, NanoString desires a judicial determination of its rights and duties with respect to any alleged infringement of the 054 Patent.

142. A judicial declaration is necessary and appropriate at this time so that the parties may proceed in accordance with their respective rights and duties determined by the Court.

ELEVENTH COUNT

(Infringement of United States Patent No. 11,377,689 by 10x)

143. NanoString incorporates by references and restates the preceding Paragraphs 141 of its Counterclaims as though fully set forth herein.

144. 10x has infringed and continues to directly infringe one or more claims of the '689 Patent, including without limitation claims 1-30, pursuant to 35 U.S.C. § 271(a), literally or under the doctrine of equivalents, by making and/or using, offering to sell, selling, and/or importing into the United States without authority all products, components, and services in connection with

10x's Visium, including without limitation Visium CytAssist, Visium Spatial Gene Expression slides, Visium Spatial Gene Expression reagents, and analysis and visualization software, Space Ranger and Loupe Browser, CSP, and other products and services sold by 10x for use in 10x's Visium workflows.

145. 10x has had knowledge of the '689 Patent since at least July 11, 2022, the date when 10x was notified of NanoString's intent to add a counterclaim of infringement of U.S. Patent 11,377,689 to the 22-cv-261 litigation. Furthermore, 10x received NanoString's proposed amended counterclaims, along with detailed infringement claim chart applying the '689 Patent to 10x's products, on July 19, 2022. On information and belief, 10x's outside counsel subsequently reviewed NanoString's amended pleadings with 10x's internal legal personnel, and 10x has affirmatively decided to continue infringing. At the very least, service of NanoString's proposed amended counterclaim on July 19, 2022 provided 10x with not just notice of the '689 Patent, but also 10x's ongoing infringement of the '689 patent. 10x's infringement since this time has been willful.

146. 10x has actively induced and continues to induce the infringement of one or more claims of the '689 Patent, including without limitation claims 1-30, pursuant to 35 U.S.C. § 271(b) through a range of activities, including without limitation making and selling Visium and related products; controlling the design, manufacture, and supply of materials, software, and instruments to be used with Visium and related products; substantially marketing Visium and related products; intentionally instructing or otherwise encouraging others, including 10x's customers and end users, to use the infringing products in the United States in the manner that infringes one or more claims of the '689 Patent; creating distribution channels for the infringing products; and supporting the sale of those products in the United States. As an example, 10x implemented Certified Service

Provider (CSP) program to promote, service, and sell the infringing products domestically. As a further example, 10x distributes Visium and related products promotional and marketing materials and Visium and related products User Manuals in websites directed to the United States Market.

147. 10x has contributed and continues to contribute to the infringement of one or more claims of the '689 Patent, including without limitation claims 1-30, pursuant to 35 U.S.C. § 271(c) through a range of activities, including without limitation, without authority, importing into the United States materials and instruments that are material components of the claimed inventions of the '689 Patent; without authority, importing into the United States materials and instruments for practicing the patented method of the '689 Patent; selling and/or offering for sale Visium and related products, or has others perform such acts on its behalf; and instructing users of 10x's Visium workflows to directly infringe one or more claims of the '689 Patent. Visium and related products are specifically designed to be used in an infringing manner, where no non-infringing use of Visium and related products has been described in 10x's instructional materials. Therefore, 10x's Visium and related products constitute a material part of the claimed invention of the '689 Patent, and are not a staple article or commodity of commerce suitable for substantial non-infringing use. As an example, 10x supplies in the United States products specifically designed for use in practicing one or more claims of the '689 Patent, including for example the Visium Spatial Gene Expression slides, Visium Spatial Gene Expression reagents, Space Ranger, and Loupe Browser and threaten to sell those products throughout the United States.

148. To demonstrate how 10x infringes one or more claims of the '689 Patent, attached is a preliminary and exemplary infringement claim chart. Ex. 2. This chart is not intended to limit NanoString's right to modify this chart or any other claim chart or allege that other activities of 10x infringes the identified claims or any other claims of the '689 Patent or any other patents. This

chart is hereby incorporated by reference in its entirety. Each claim element that is mapped to Visium and related products shall be considered an allegation within the meaning of the Federal Rules of Civil Procedure and therefore a response to each allegation is required.

149. NanoString has suffered and continues to suffer damages as a result of 10x's infringement of the '689 Patent.

150. Unless 10x is enjoined from infringing the '689 Patent, 10x's efforts to design, develop, market, offer to sell, and sell Visium and related products will cause NanoString to suffer irreparable injury for which damages are an inadequate remedy.

REQUEST FOR RELIEF

WHEREFORE, having fully answered Plaintiffs' FAC and having asserted Affirmative Defenses, and Counterclaims, NanoString respectfully requests the following relief:

A. That this Court enter judgment on Plaintiffs' FAC and NanoString's Counterclaims in favor of NanoString, against Plaintiffs, with Plaintiffs being awarded no relief of any kind in this action;

B. That this Court enter judgment and/or declarations that NanoString does not infringe the Asserted Patents and that the Asserted Patents are invalid;

C. That this Court enter judgement that Counterclaim-Defendant 10x has infringed and continues to infringe one or more claims of the '689 Patent, either literally or under the doctrine of equivalents;

D. That this Court enter a declaration that the '689 Patent is valid and enforceable;

E. That this Court enter an order preliminarily and permanently enjoining Counterclaims-Defendant 10x, and its officers, directors, agents, servants, affiliates, employees, divisions, branches, subsidiaries, parents, assigns and successors in interest, and all others acting in active concert therewith, including related individuals and entities, customers, representatives,

distributors, and dealers, from further infringement of the '689 Patent. In the alternative, if the Court finds that an injunction is not warranted, Counterclaim-Plaintiff NanoString requests an award of post-judgement royalty to compensate for future infringement;

F. An award of all monetary relief adequate to compensate for damages resulting from 10x's infringement, including lost profits but in no event less than a reasonable royalty under 35 U.S.C. § 284 for 10x's infringement, including all pre-judgement and post-judgement interest at the maximum rate allowed by law;

G. That this Court enter a judgment declaring this case exceptional under 35 U.S.C. § 285 and awarding NanoString its attorneys' fees and prejudgment and post-judgment interest;H.

That this Court award NanoString all of its costs of this action; and

I. That this Court grant such other and further relief as the Court shall deem just and proper.

Respectfully submitted,

FARNAN LLP

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Date: July 29, 2022

Exhibit 1

US011377689B2

(12) United States Patent

Beechem et al.

(10) Patent No.: US 11,377,689 B2
(45) Date of Patent: *Jul. 5, 2022

(54) **CHEMICAL COMPOSITIONS AND USES THEREOF**

(71) Applicant: **NanoString Technologies, Inc.**, Seattle, WA (US)

(72) Inventors: **Joseph M. Beechem**, Eugene, OR (US); **Dae Kim**, Bellevue, WA (US); **Margaret Hoang**, Seattle, WA (US); **Mark Gregory**, Boise, ID (US); **Erin Piazza**, Edmonds, WA (US); **Denise Zhou**, Seattle, WA (US)

(73) Assignee: **NanoString Technologies, Inc.**, Seattle, WA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **17/476,707**

(22) Filed: **Sep. 16, 2021**

(65) **Prior Publication Data**

US 2021/0403998 A1 Dec. 30, 2021

Related U.S. Application Data

(63) Continuation of application No. 16/272,487, filed on Feb. 11, 2019, now abandoned.
(Continued)

(51) **Int. Cl.**
C12P 19/34 (2006.01)
C12Q 1/6876 (2018.01)
(Continued)

(52) **U.S. Cl.**
CPC ***C12Q 1/6876*** (2013.01); ***C12Q 1/6804*** (2013.01); ***C12Q 1/686*** (2013.01);
(Continued)

(58) **Field of Classification Search**
USPC 435/6.1, 6.11, 6.12, 91.1, 91.2, 91.51, 435/283.1, 287.1, 287.2; 436/94, 501;
(Continued)

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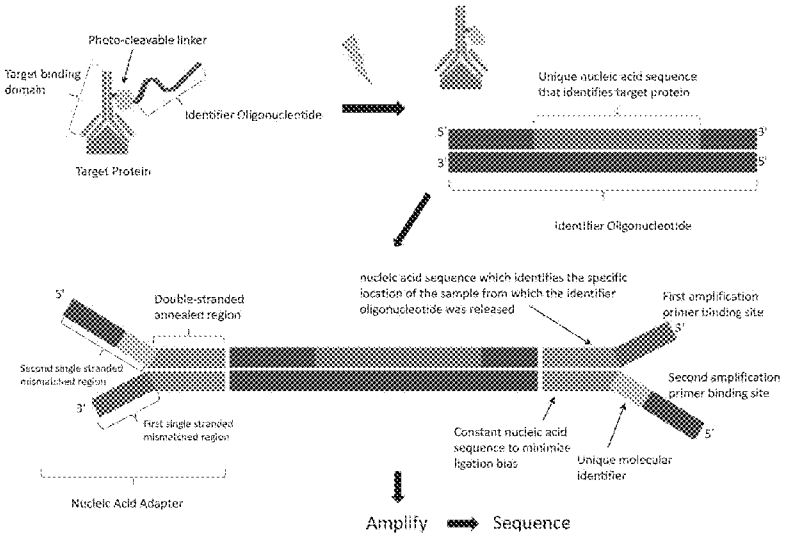
Primary Examiner — Frank W Lu
(74) *Attorney, Agent, or Firm* — Cooley LLP; Ivor R. Elrifi; Matthew Pavao

(57) **ABSTRACT**

The present invention relates to, among other things, probes, compositions, methods, and kits for simultaneous, multiplexed detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell that are adaptable for use with existing sequencing technologies.

30 Claims, 40 Drawing Sheets
(29 of 40 Drawing Sheet(s) Filed in Color)
Specification includes a Sequence Listing.



Related U.S. Application Data

(60) Provisional application No. 62/771,212, filed on Nov. 26, 2018, provisional application No. 62/629,180, filed on Feb. 12, 2018.

(51) **Int. Cl.**

C12Q 1/6818	(2018.01)
C12Q 1/6858	(2018.01)
C12Q 1/6848	(2018.01)
C12Q 1/6869	(2018.01)
C12Q 1/686	(2018.01)
C12Q 1/6851	(2018.01)
C12Q 1/6855	(2018.01)
C12Q 1/6841	(2018.01)
C12Q 1/6804	(2018.01)

(52) **U.S. Cl.**

CPC ***C12Q 1/6818*** (2013.01); ***C12Q 1/6841*** (2013.01); ***C12Q 1/6848*** (2013.01); ***C12Q 1/6851*** (2013.01); ***C12Q 1/6855*** (2013.01); ***C12Q 1/6858*** (2013.01); ***C12Q 1/6869*** (2013.01); *C12Q 2600/16* (2013.01)

(58) **Field of Classification Search**

USPC 536/23.1, 24.3, 24.33, 25.3

See application file for complete search history.

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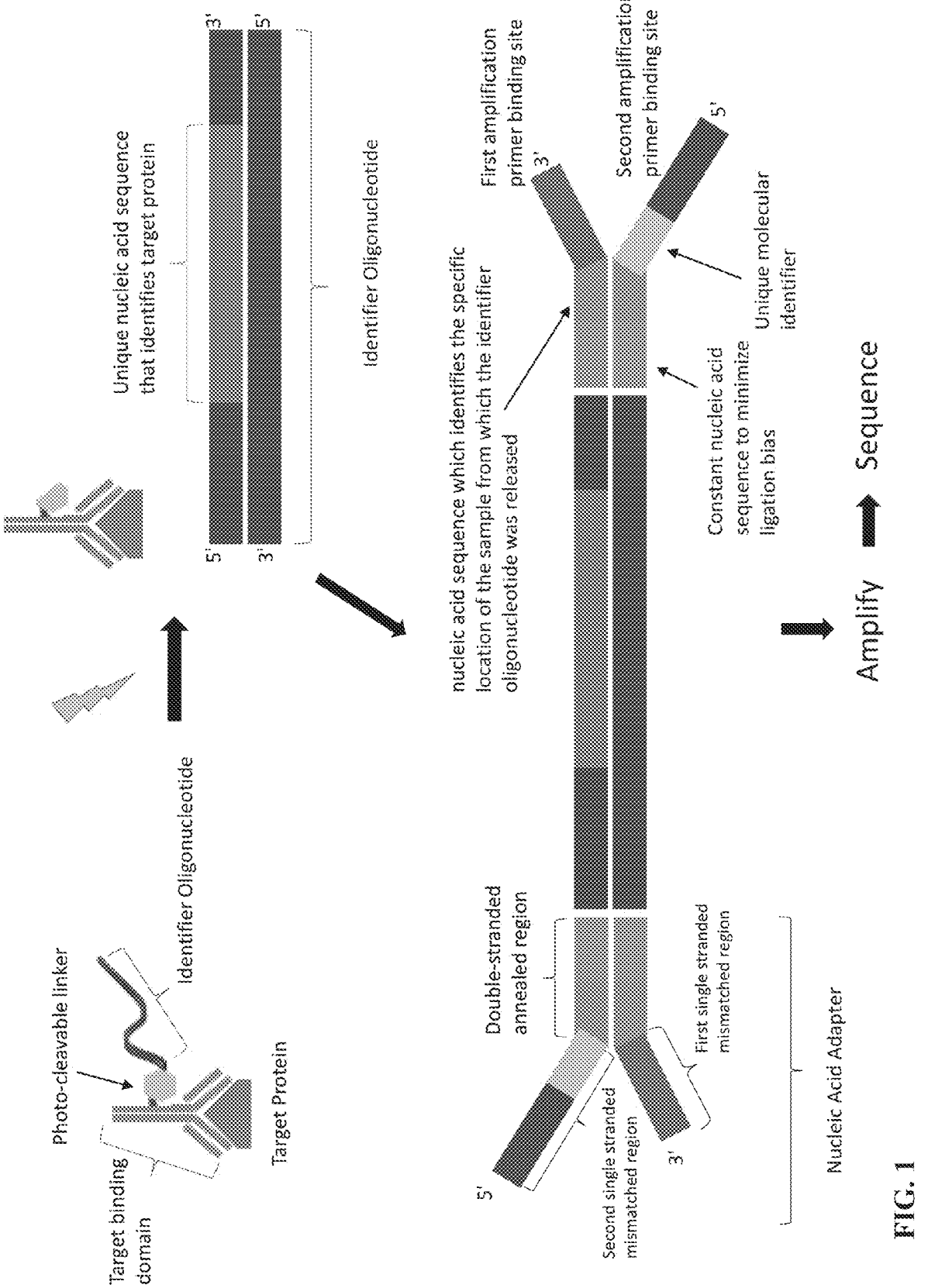
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Identifier Oligonucleotide
Target binding domain
Target Protein
Unique nucleic acid sequence that identifies target protein
Identifier Oligonucleotide
5'
3'
3'
5'
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First amplification primer binding site
Second amplification primer binding site
Constant nucleic acid sequence to minimize ligation bias
Unique molecular identifier
Double-stranded annealed region
First single stranded mismatched region
Second single stranded mismatched region
Nucleic Acid Adapter
Amplify
Sequence

FIG. 1

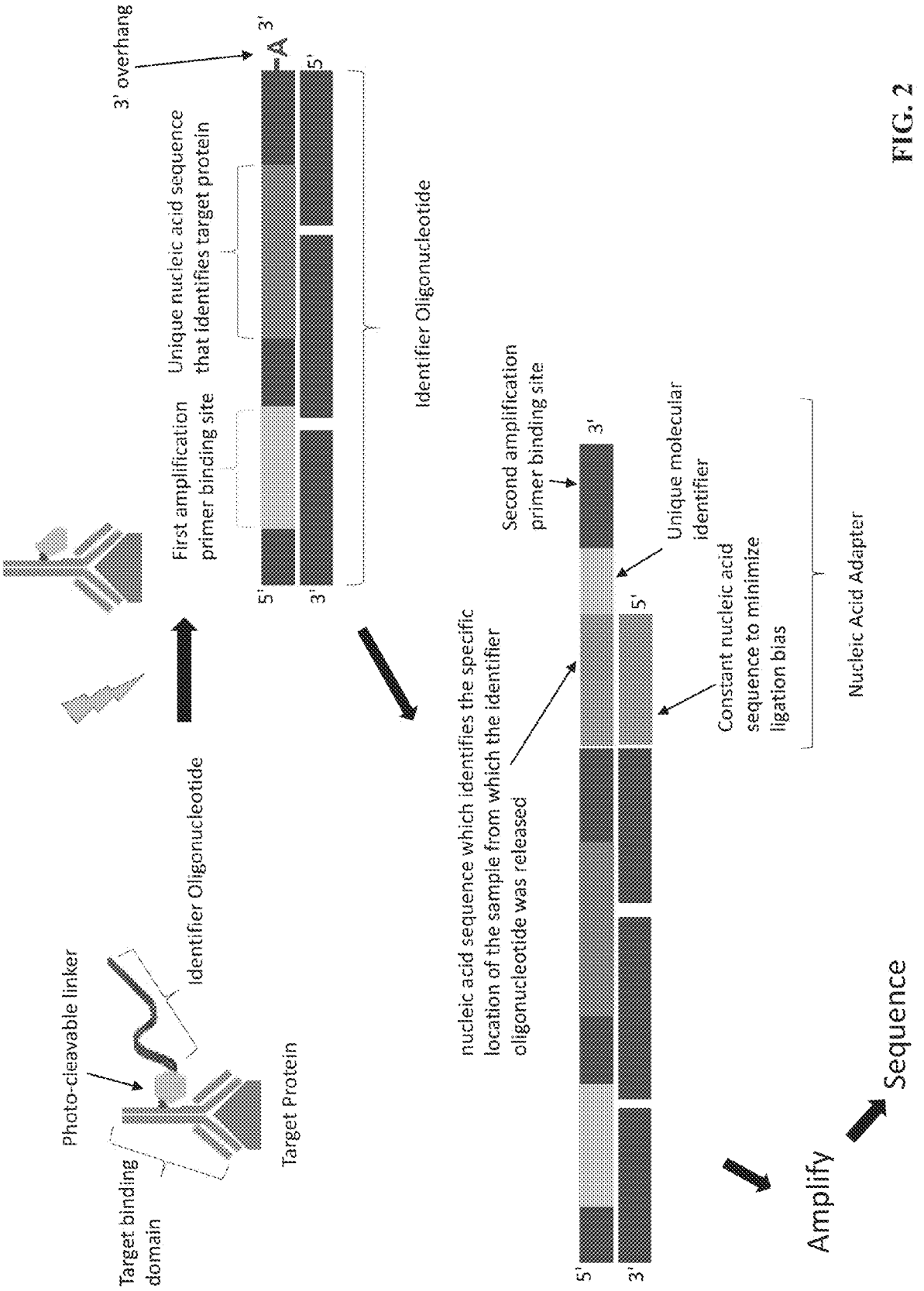


Photo-cleavable linker
Identifier Oligonucleotide
Target binding domain
Target Protein
3' overhang
A
3'
5'
First amplification primer binding site
Unique nucleic acid sequence that identifies target protein
Identifier Oligonucleotide
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Second amplification primer binding site
Unique molecular identifier
Constant nucleic acid sequence to minimize ligation bias
Nucleic Acid Adapter
Amplify
Sequence

FIG. 2

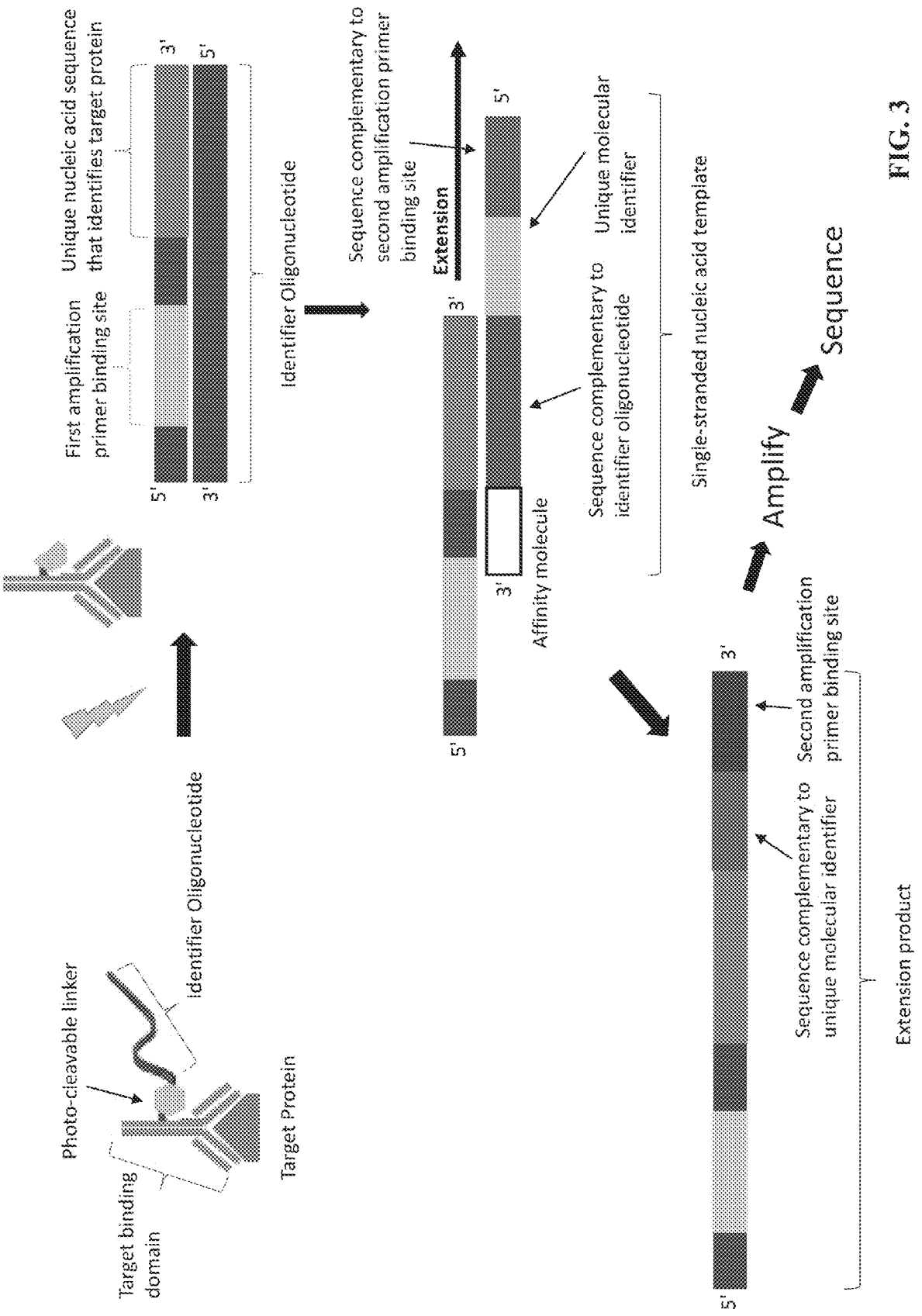


Photo-cleavable linker
Identifier Oligonucleotide
Target binding domain
Target Protein
Identifier Oligonucleotide
First amplification primer binding site
Unique nucleic acid sequence that identifies target protein
5'
3'
3'
5'
Sequence complementary to second amplification primer binding site
Extension
3'
5'
Affinity molecule
Sequence complementary to identifier oligonucleotide
Unique molecular identifier
Single-stranded nucleic acid template
Amplify
Sequence
5'
3'
Sequence complementary to unique molecular identifier
Second amplification primer binding site
Extension product

FIG. 3

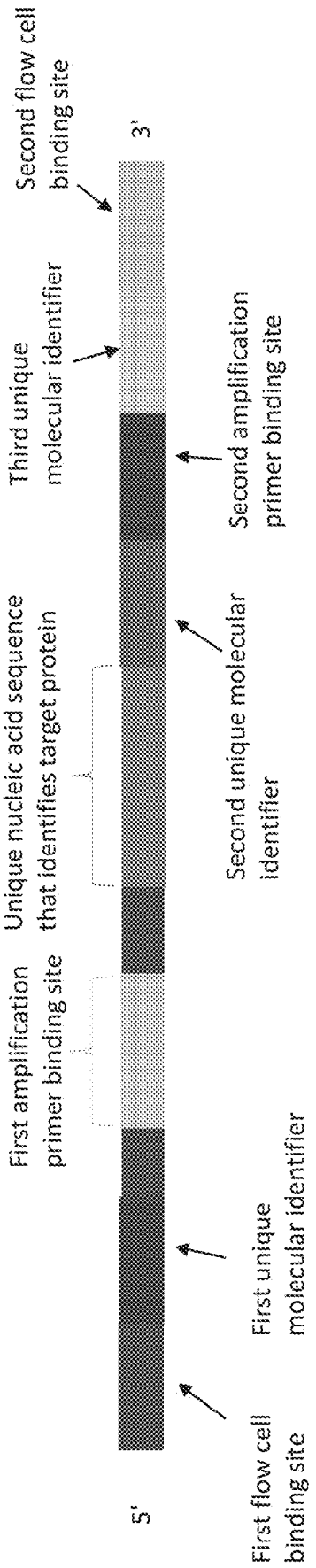

Second flow cell binding site
3'
Third unique molecular identifier
Second amplification primer binding site
Unique nucleic acid sequence that identifies target protein
Second unique molecular identifier
First amplification primer binding site
First unique molecular identifier
5'
First flow cell binding site

FIG. 4

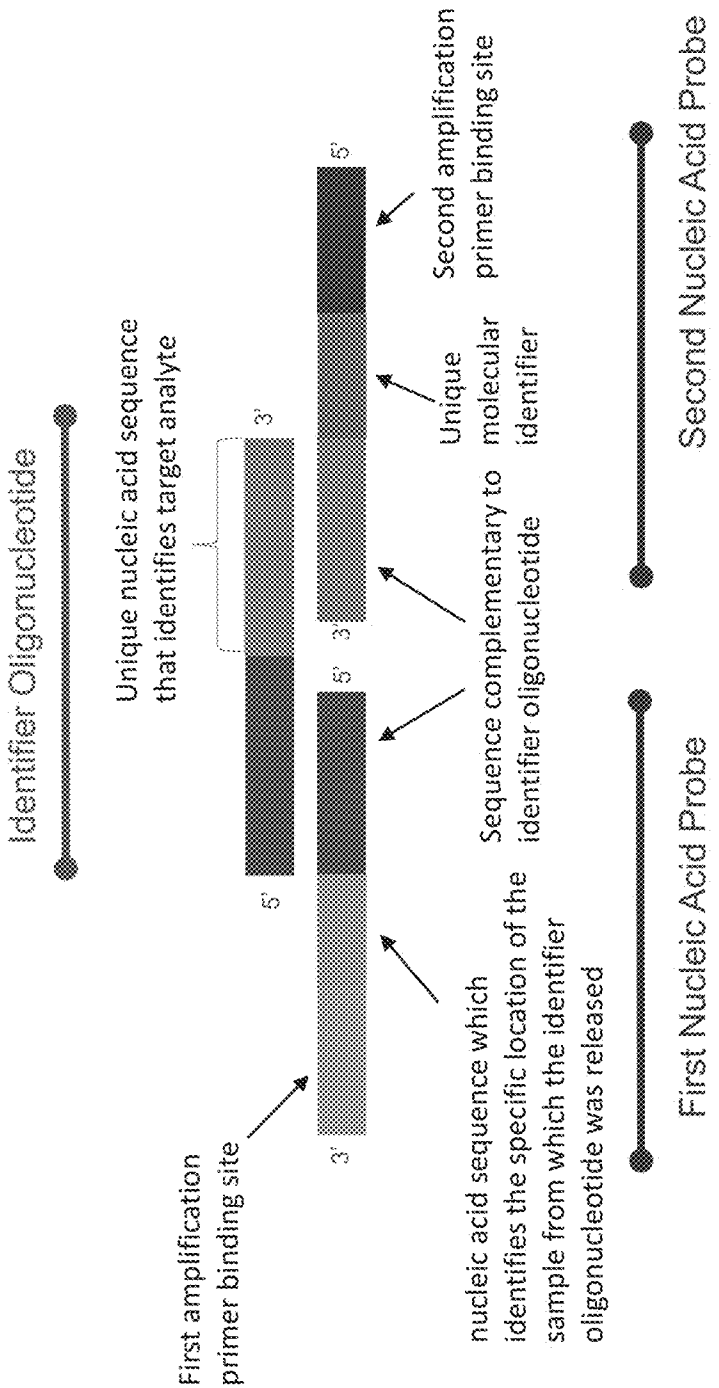

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
Second amplification primer binding site
Unique molecular identifier
Sequence complementary to identifier oligonucleotide
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First amplification primer binding site
First Nucleic Acid Probe
Second Nucleic Acid Probe
5'
3'

FIG. 5

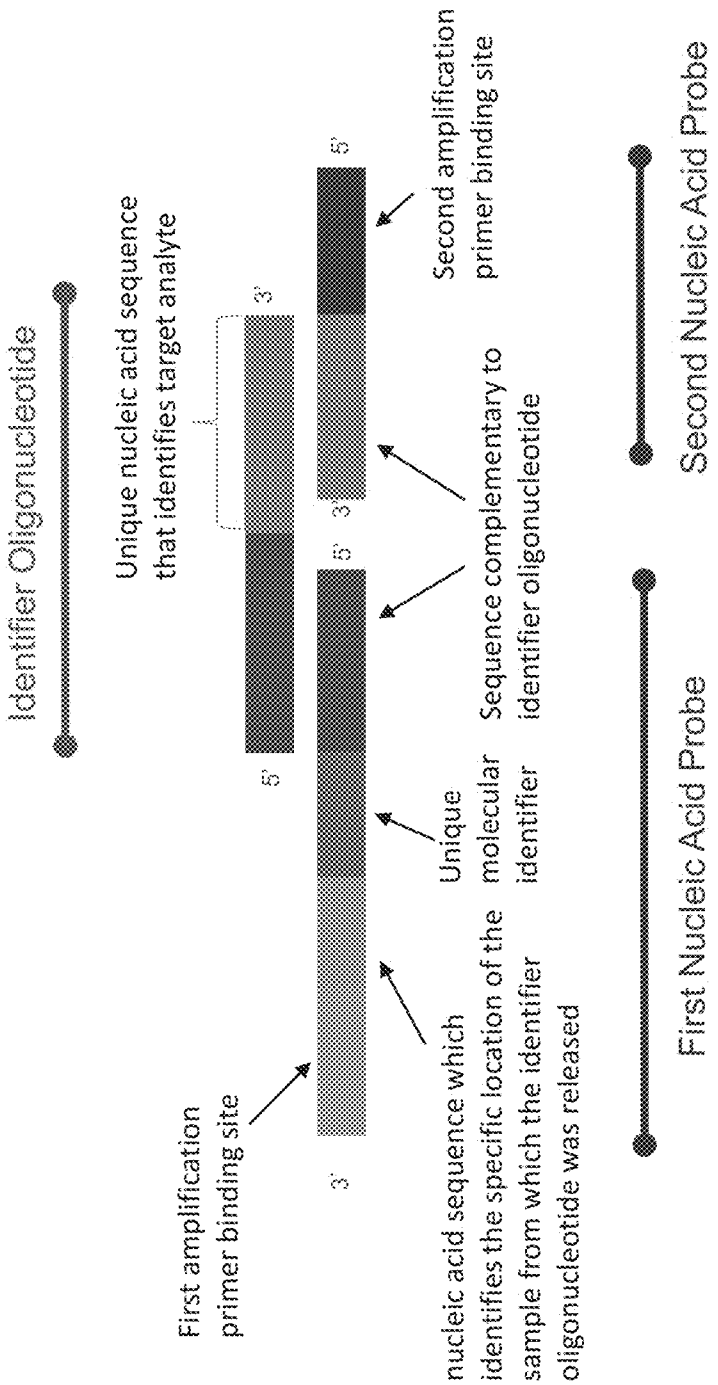

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
Second amplification primer binding site
Sequence complementary to identifier oligonucleotide
Unique molecular identifier
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First amplification primer binding site
First Nucleic Acid Probe
Second Nucleic Acid Probe
5'
3'

FIG. 6

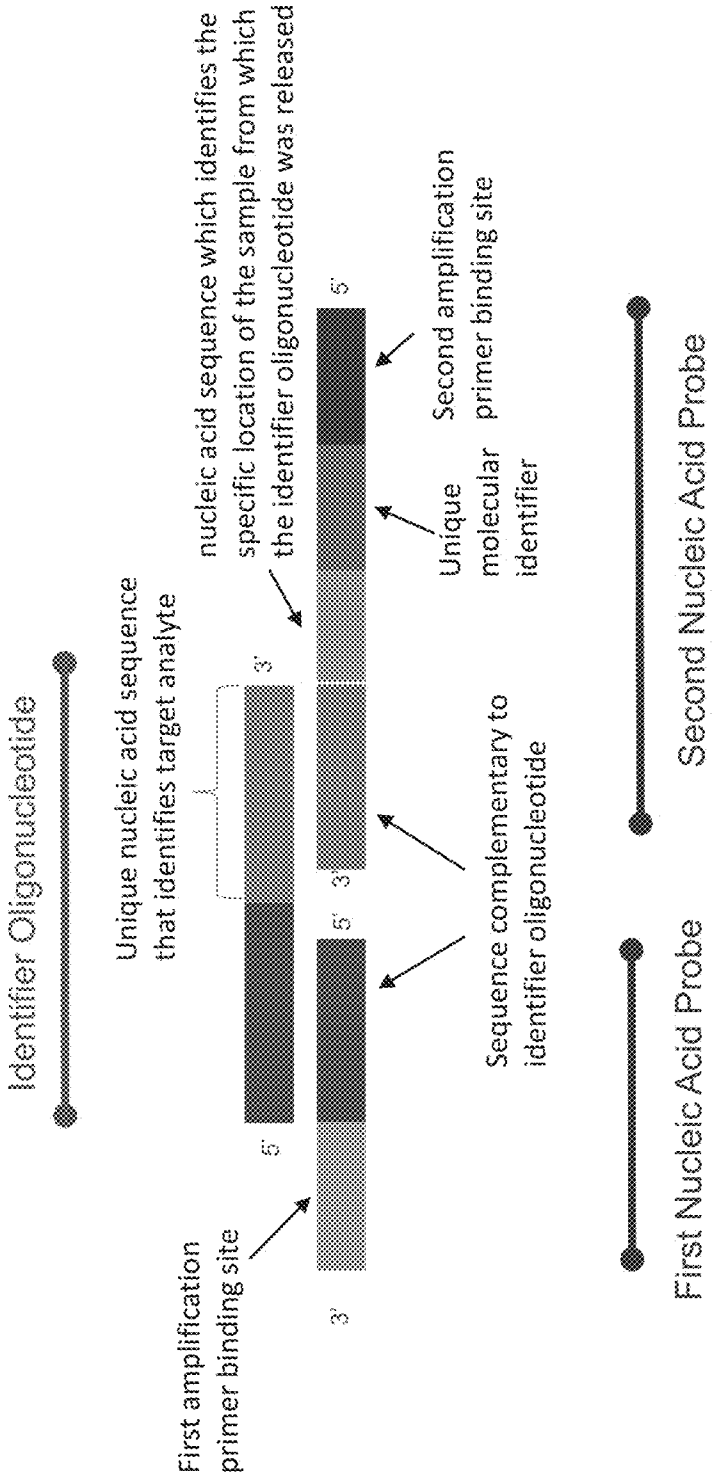

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Second amplification primer binding site
Unique molecular identifier
Sequence complementary to identifier oligonucleotide
First amplification primer binding site
First Nucleic Acid Probe
Second Nucleic Acid Probe
5'
3'
5'
3'
5'
3'

FIG. 7

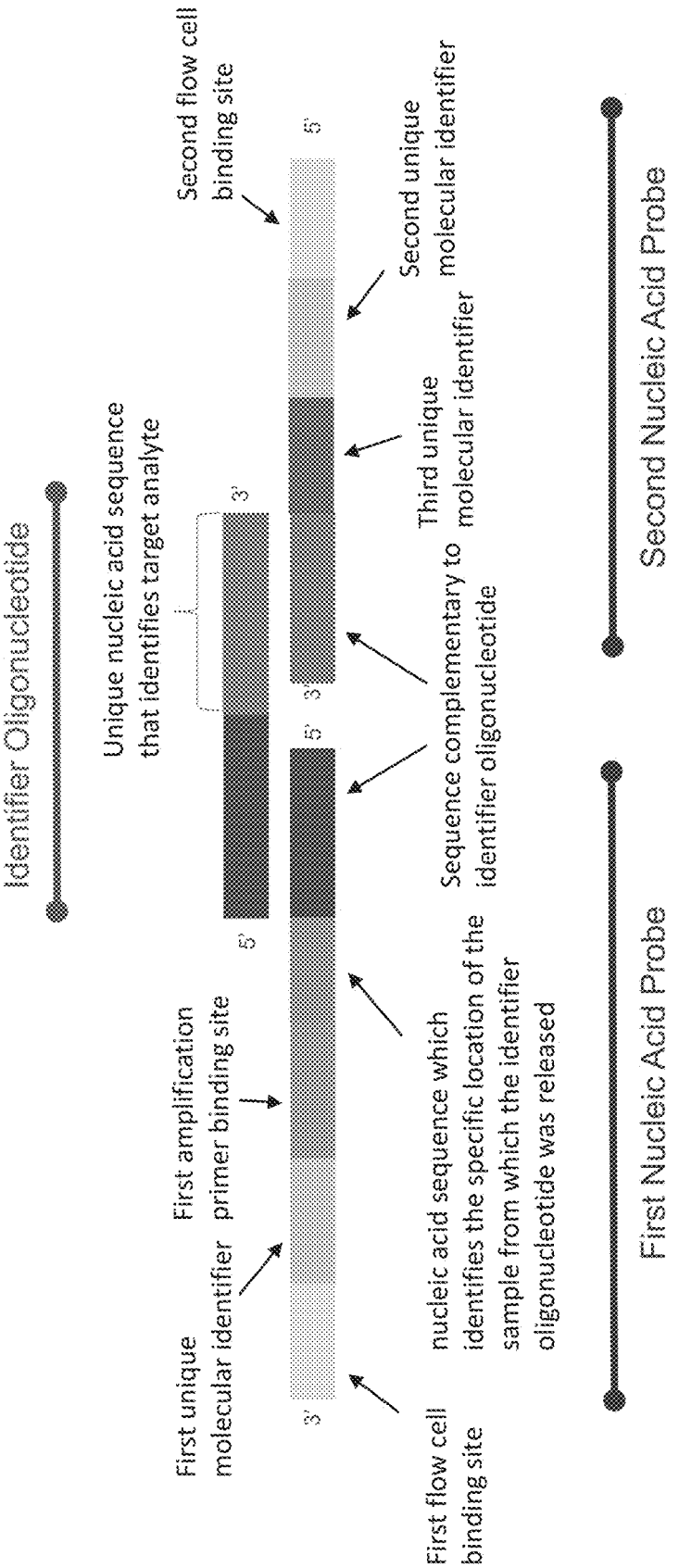

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
Second flow cell binding site
Second unique molecular identifier
Third unique molecular identifier
Sequence complementary to identifier oligonucleotide
First amplification primer binding site
First unique molecular identifier
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First flow cell binding site
First Nucleic Acid Probe
Second Nucleic Acid Probe
5'
3'

FIG. 8

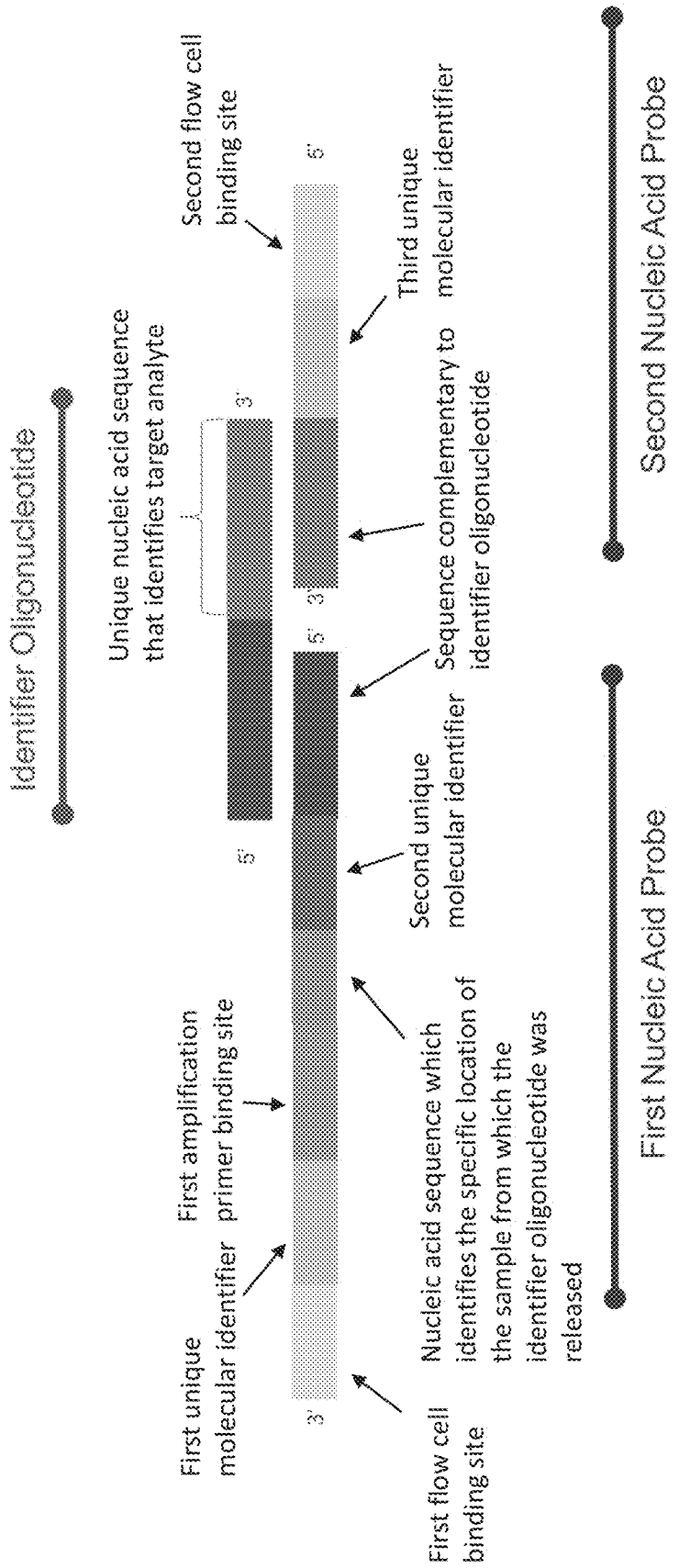

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
Second flow cell binding site
Third unique molecular identifier
Sequence complementary to identifier oligonucleotide
Second unique molecular identifier
First amplification primer binding site
First unique molecular identifier
Nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First flow cell binding site
First Nucleic Acid Probe
Second Nucleic Acid Probe
5'
3'

FIG. 9

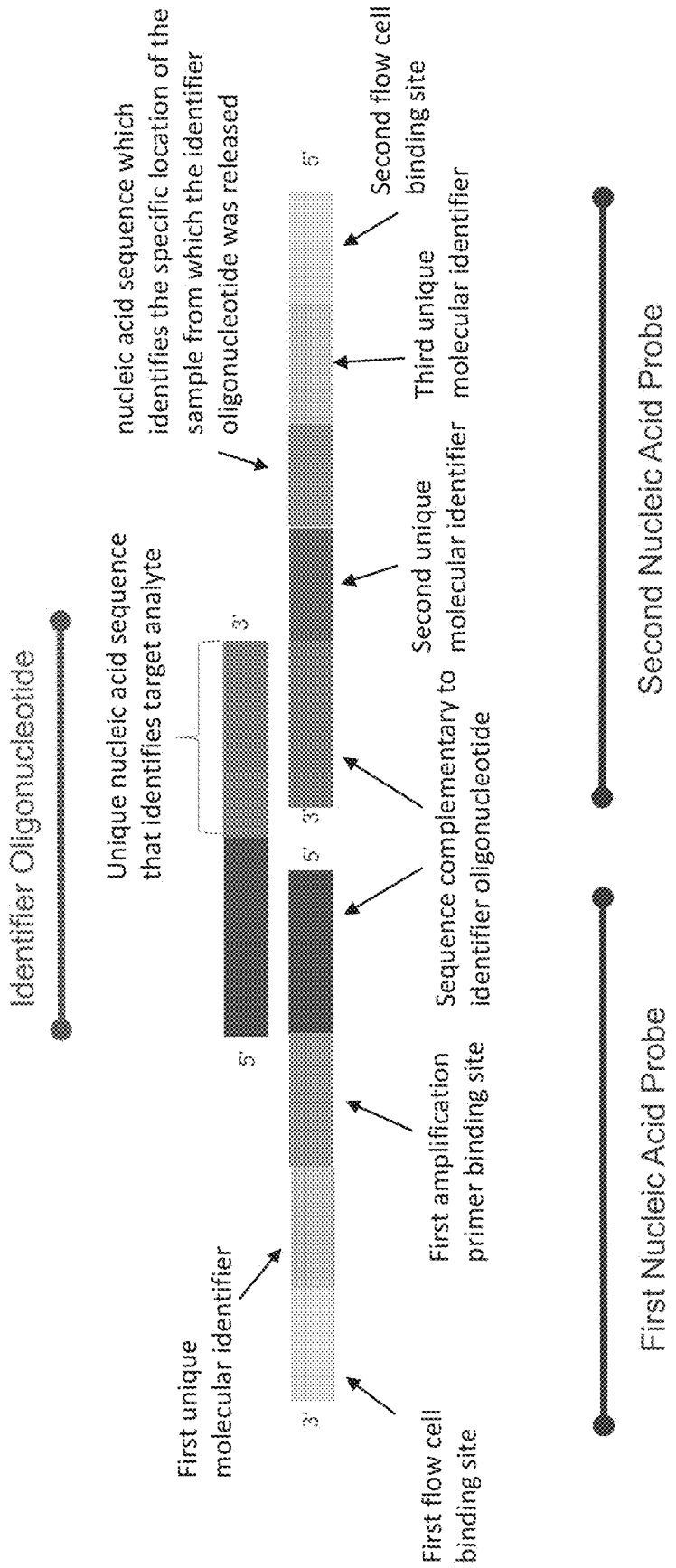

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Second flow cell binding site
Third unique molecular identifier
Second unique molecular identifier
Sequence complementary to identifier oligonucleotide
First amplification primer binding site
First unique molecular identifier
First flow cell binding site
5'
3'
First Nucleic Acid Probe
Second Nucleic Acid Probe

FIG. 10

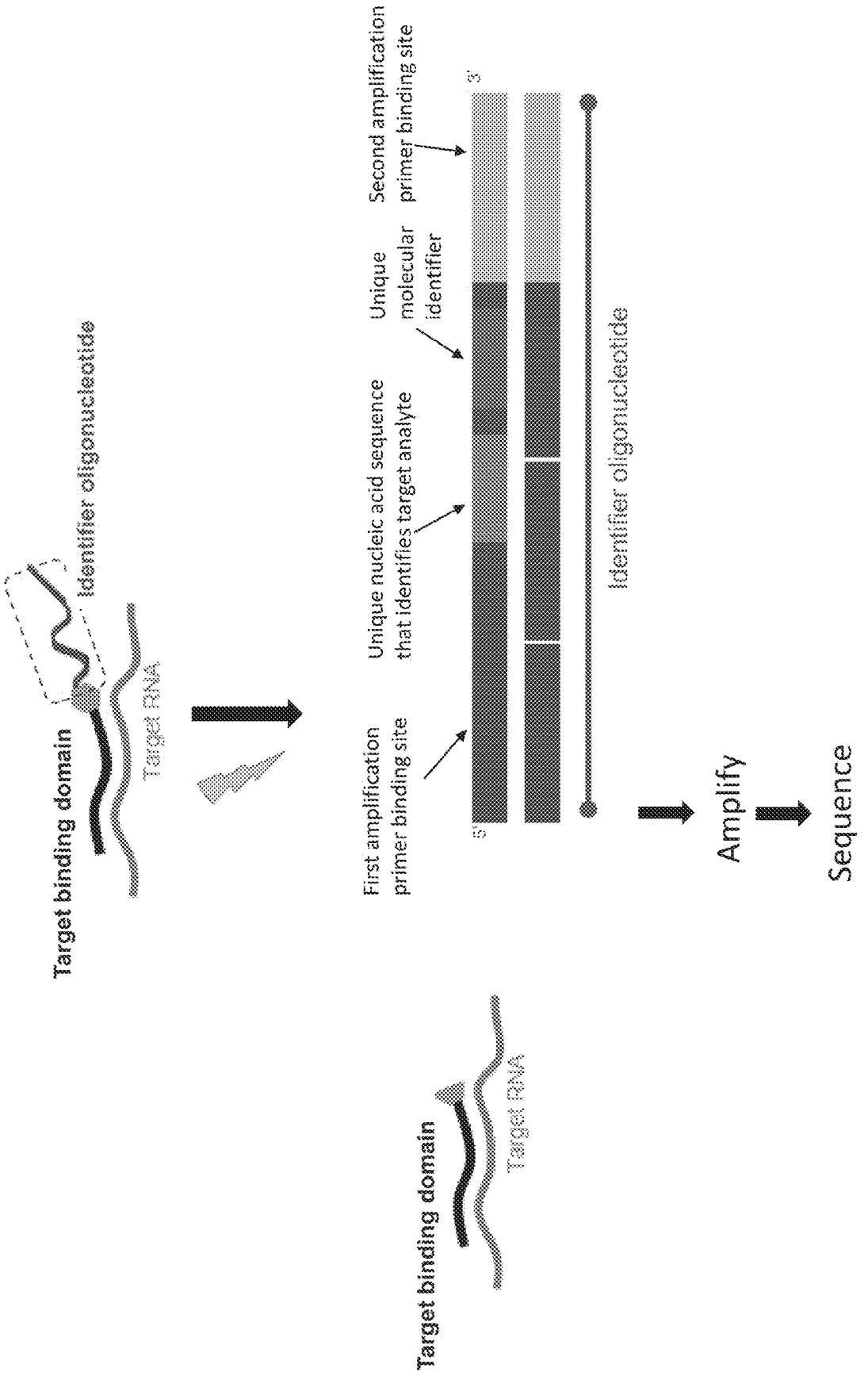

Target binding domain
Identifier oligonucleotide
Target RNA
First amplification primer binding site
Unique nucleic acid sequence that identifies target analyte
Unique molecular identifier
Second amplification primer binding site
5'
3'
Identifier oligonucleotide
Amplify
Sequence
Target binding domain
Target RNA

FIG. 11

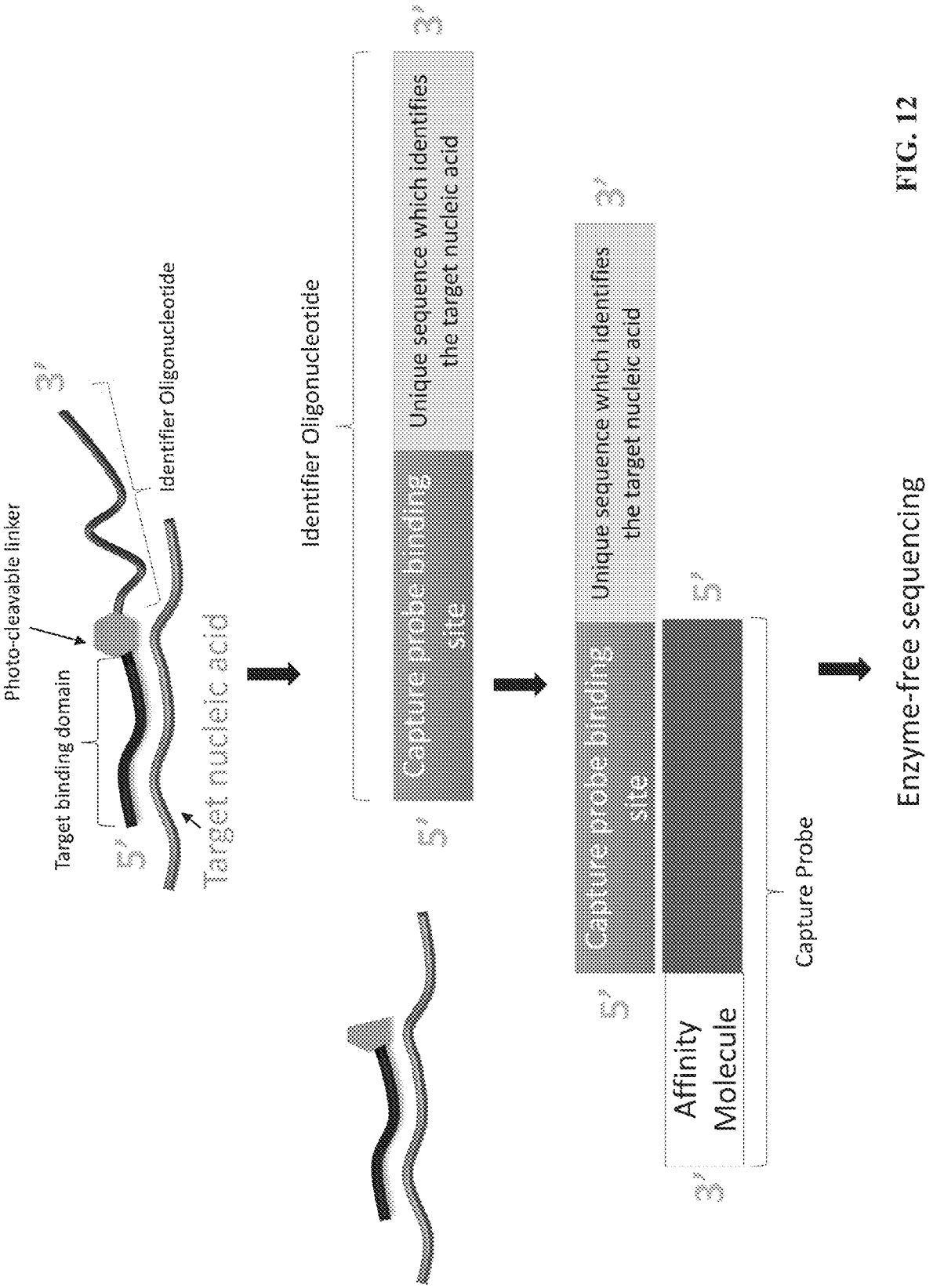


Photo-cleavable linker
Target binding domain
Identifier Oligonucleotide
3'
5'
Target nucleic acid
Identifier Oligonucleotide
Capture probe-binding site
Unique sequence which identifies the target nucleic acid
3'
5'
Capture probe binding Site
Unique sequence which identifies the target nucleic acid
3'
5'
Affinity Molecule
3'
Capture Probe
Enzyme-free sequencing

FIG. 12

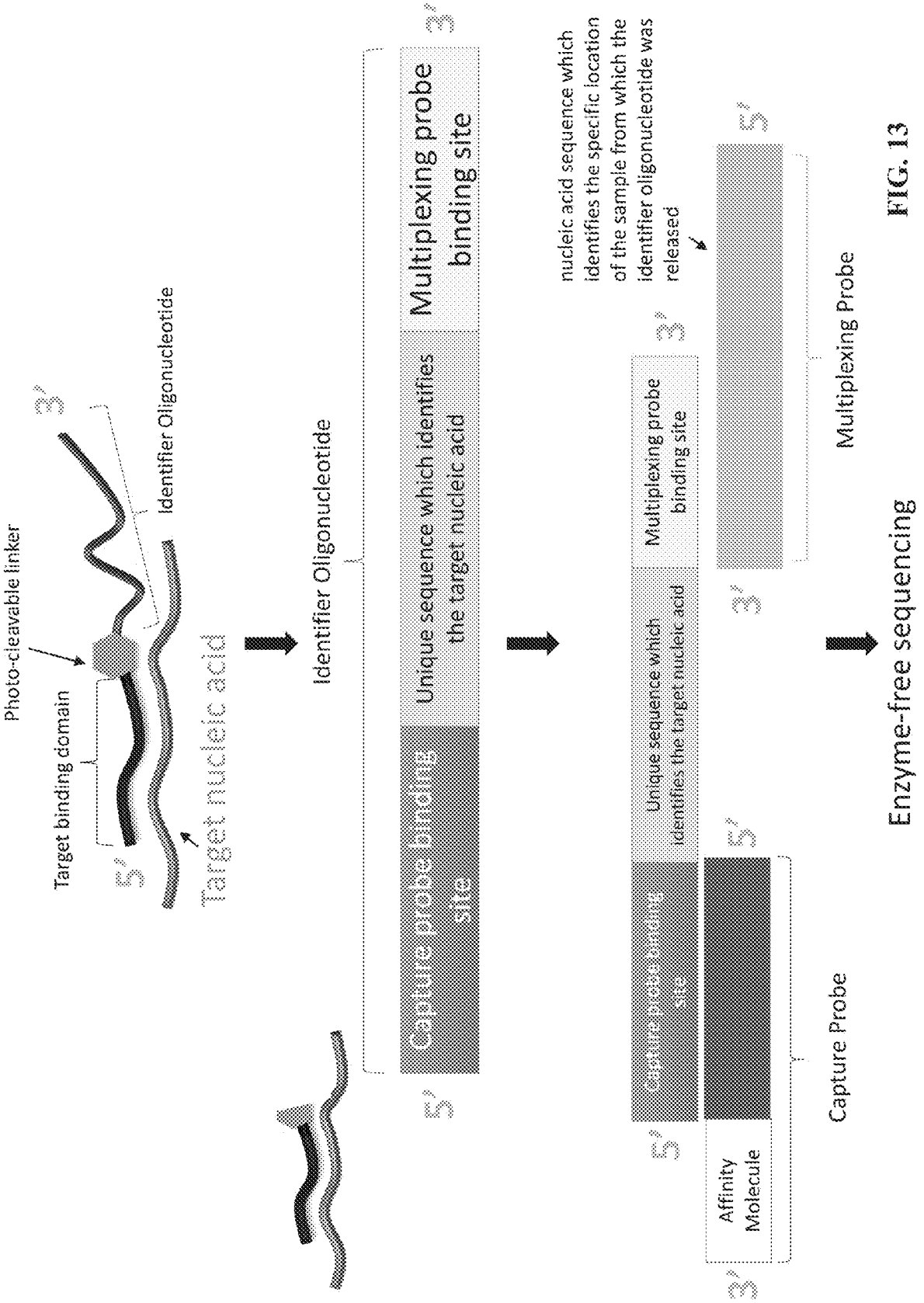


Photo-cleavable linker
Target binding domain
Identifier Oligonucleotide
3'
5'
Target nucleic acid
Identifier Oligonucleotide
Capture probe binding site
Unique sequence which identifies the target nucleic acid
Multiplexing probe binding site
5'
3'
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Capture probe binding site
Unique sequence which identifies the target nucleic acid
Multiplexing probe binding site
Affinity Molecule
Capture Probe
Multiplexing Probe
Enzyme-free sequencing

FIG. 13

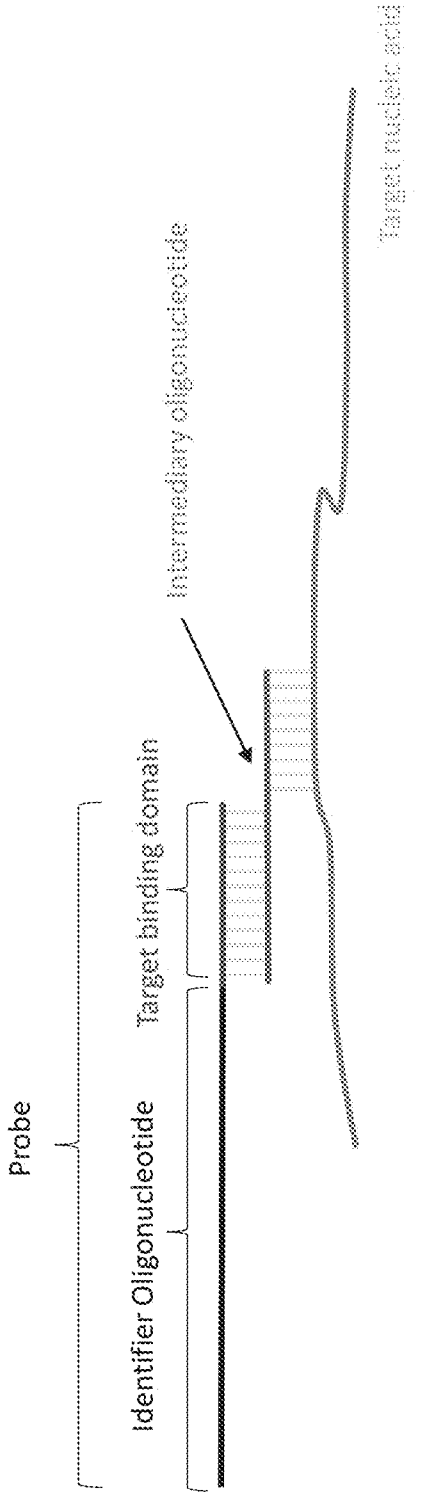

Target nucleic acid
Intermediary oligonucleotide
Target binding domain
Identifier Oligonucleotide
Probe

FIG. 14

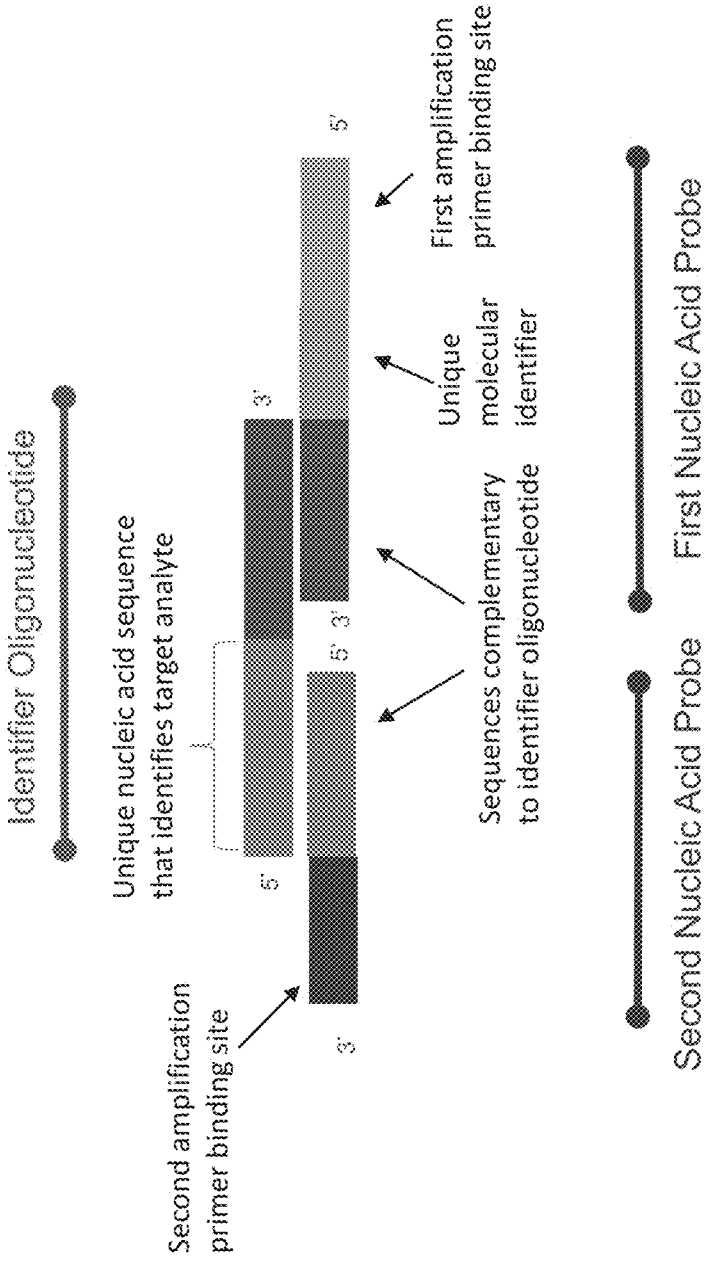

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
Second amplification primer binding site
Sequences complementary to identifier oligonucleotide
Unique molecular identifier
First amplification primer binding site
Second Nucleic Acid Probe
First Nucleic Acid Probe
5'
3'
5'
3'
5'
3'

FIG. 15

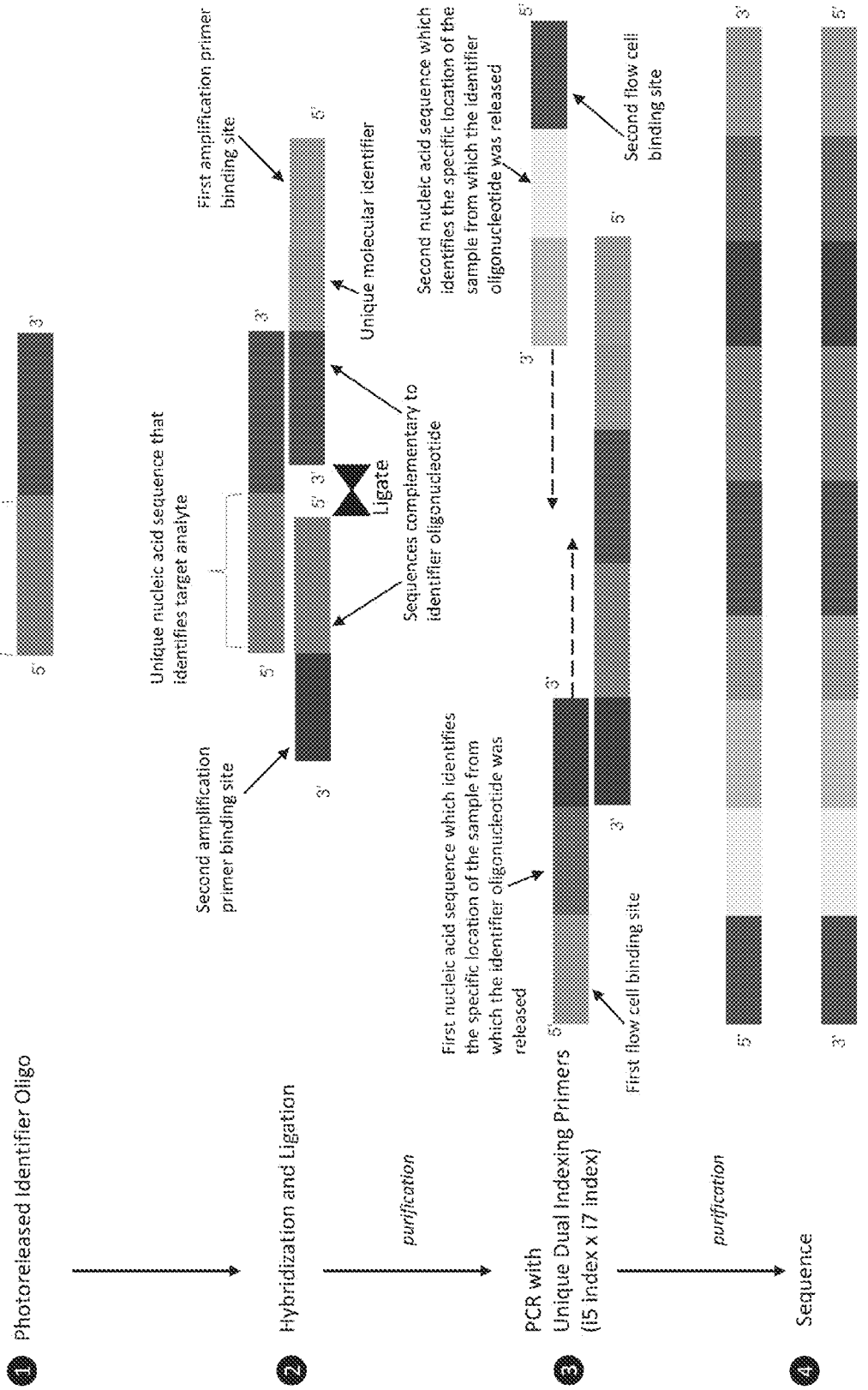

1 Photoreleased Identifier Oligo
Unique nucleic acid sequence that identifies target analyte
5'
3'
2 Hybridization and Ligation
Unique nucleic acid sequence that identifies target analyte
First amplification primer binding site
Unique molecular identifier
Sequences complementary to identifier oligonucleotide
Ligate
Second amplification primer binding site
purification
3 PCR with Unique Dual Indexing Primers (i5 index x i7 index)
First nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Second nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First flow cell binding site
Second flow cell binding site
purification
4 Sequence

FIG. 16

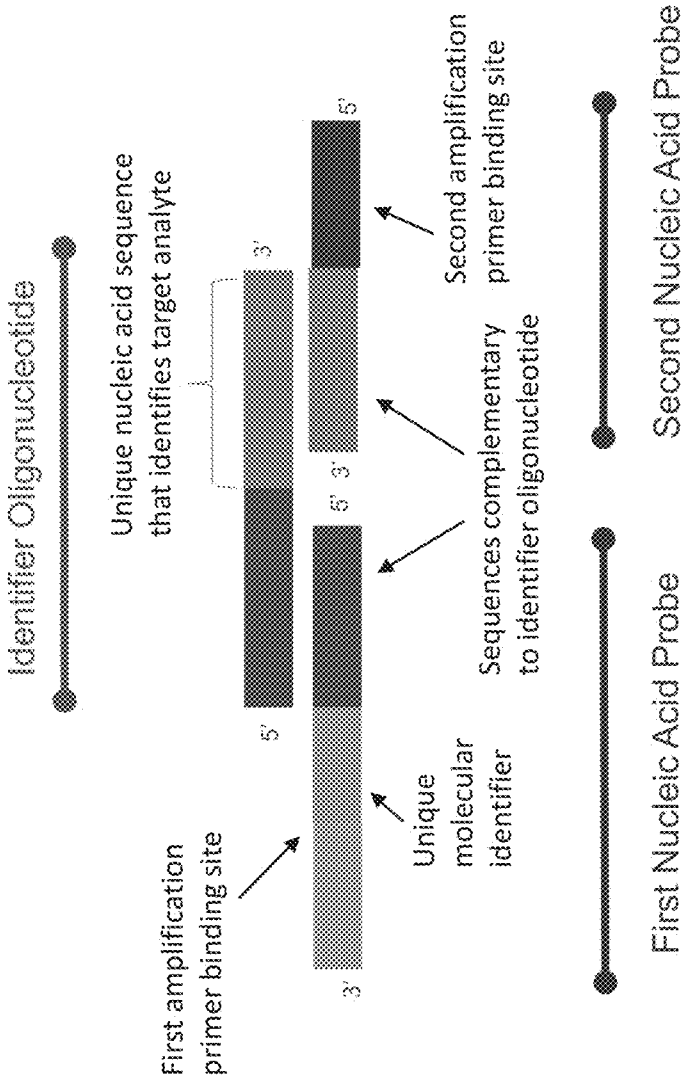

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
First amplification primer binding site
Unique molecular identifier
Sequences complementary to identifier oligonucleotide
Second amplification primer binding site
First Nucleic Acid Probe
Second Nucleic Acid Probe
5'
3'
5'
3'
5'
3'

FIG. 17

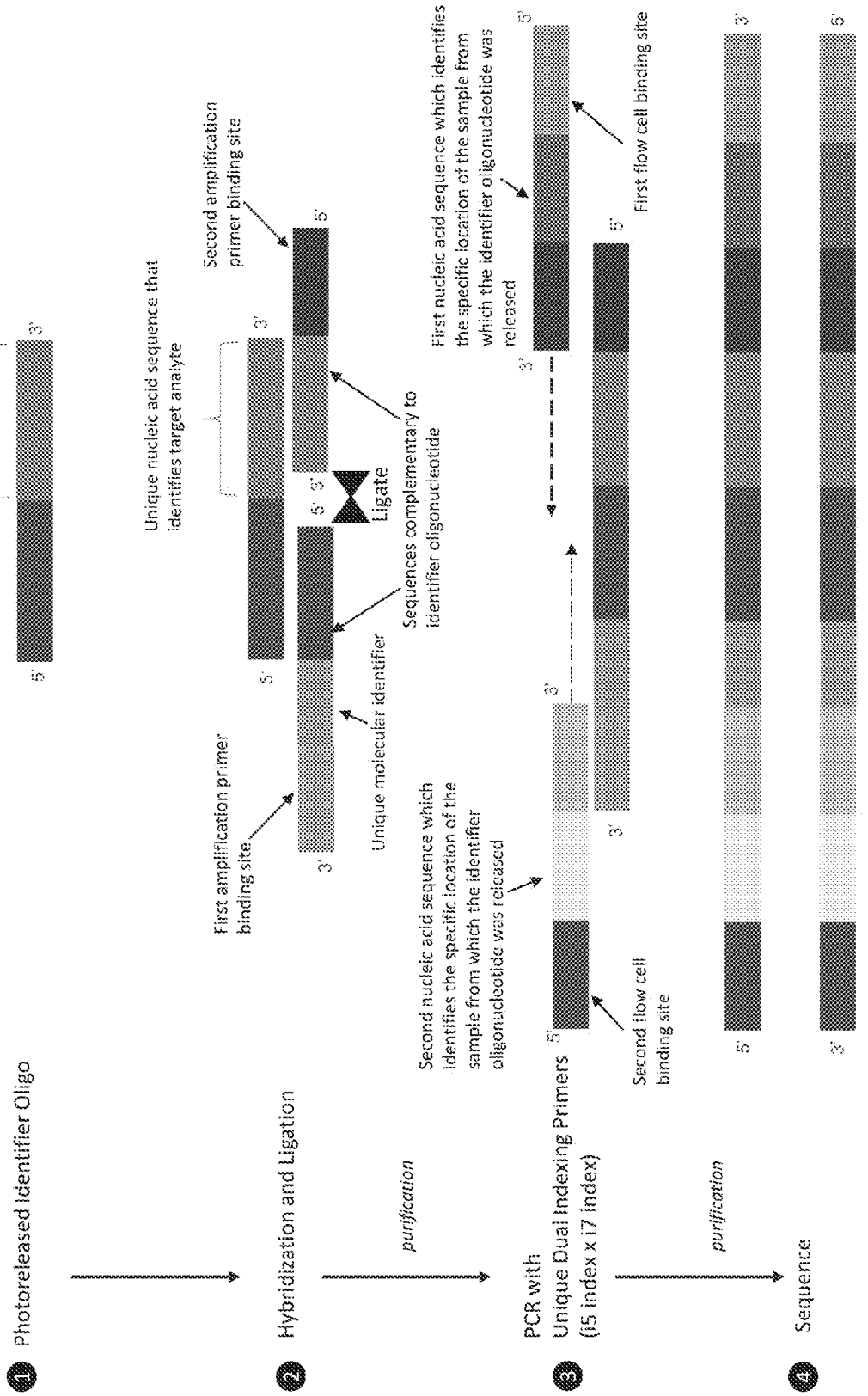

1 Photoreleased Identifier Oligo
Unique nucleic acid sequence that identifies target analyte
5'
3'
2 Hybridization and Ligation
Unique nucleic acid sequence that identifies target analyte
Second amplification primer binding site
First amplification primer binding site
Unique molecular identifier
Sequences complementary to identifier oligonucleotide
Ligate
purification
3 PCR with Unique Dual Indexing Primers (i5 index x i7 index)
Second nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First flow cell binding site
Second flow cell binding site
purification
4 Sequence

FIG. 18

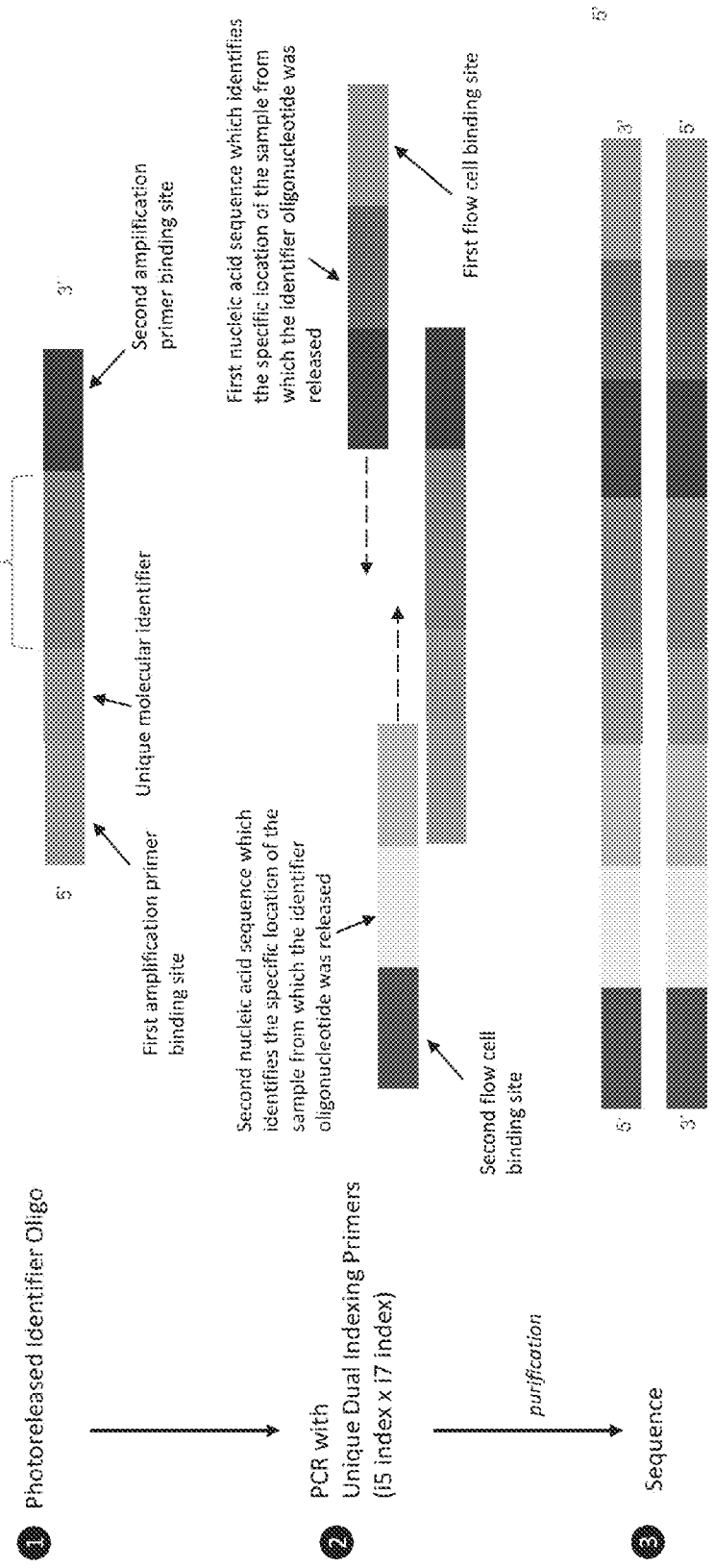

1 Photoreleased Identifier Oligo
Unique nucleic acid sequence that identifies target analyte
Second amplification primer binding site
Unique molecular identifier
First amplification primer binding site
5'
3'
2 PCR with Unique Dual Indexing Primers (i5 index x i7 index)
Second nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First flow cell binding site
Second flow cell binding site
purification
3 Sequence
5'
3'
5'
3'

FIG. 19

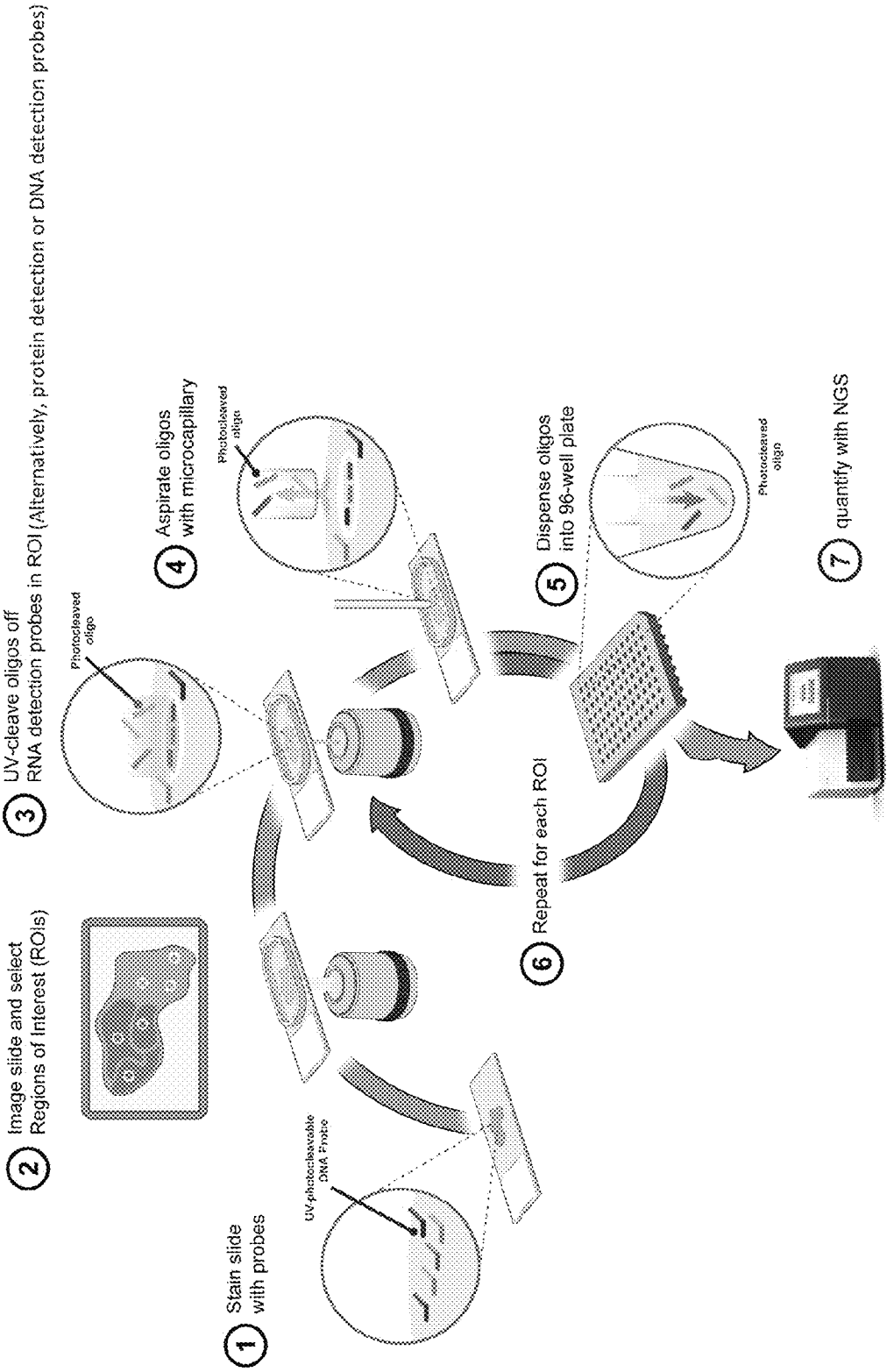

1 Stain slide with probes
UV-photocleavable DNA Probe
2 Image slide and select Regions of Interest (ROIs)
3 UV-cleave oligos off RNA detection probes in ROI (Alternatively, protein detection or DNA detection probes)
Photocleaved oligo
4 Aspirate oligos with microcapillary
Photocleaved oligo
5 Dispense oligos into 96-well plate
Photocleaved oligo
6 Repeat for each ROI
7 quantify with NGS

FIG. 20

Protein Immuno-oncology Panel (30-plex)
AKT
B7-H3
Bcl-2
Beta-2-microglobulin
Beta-Catenin
CD14
CD19
CD3
CD4
CD44
CD45
CD45RO
CD56
CD68
CD8A
FOXP3
GZMB
Histone H3
Ki67
CD20
P-AKT
PanCK
PD1
PD-L1
S6
STAT3
P-STAT3
VISTA
IgG Rabbit isotype control
IgG Mouse isotype control

FIG. 21A

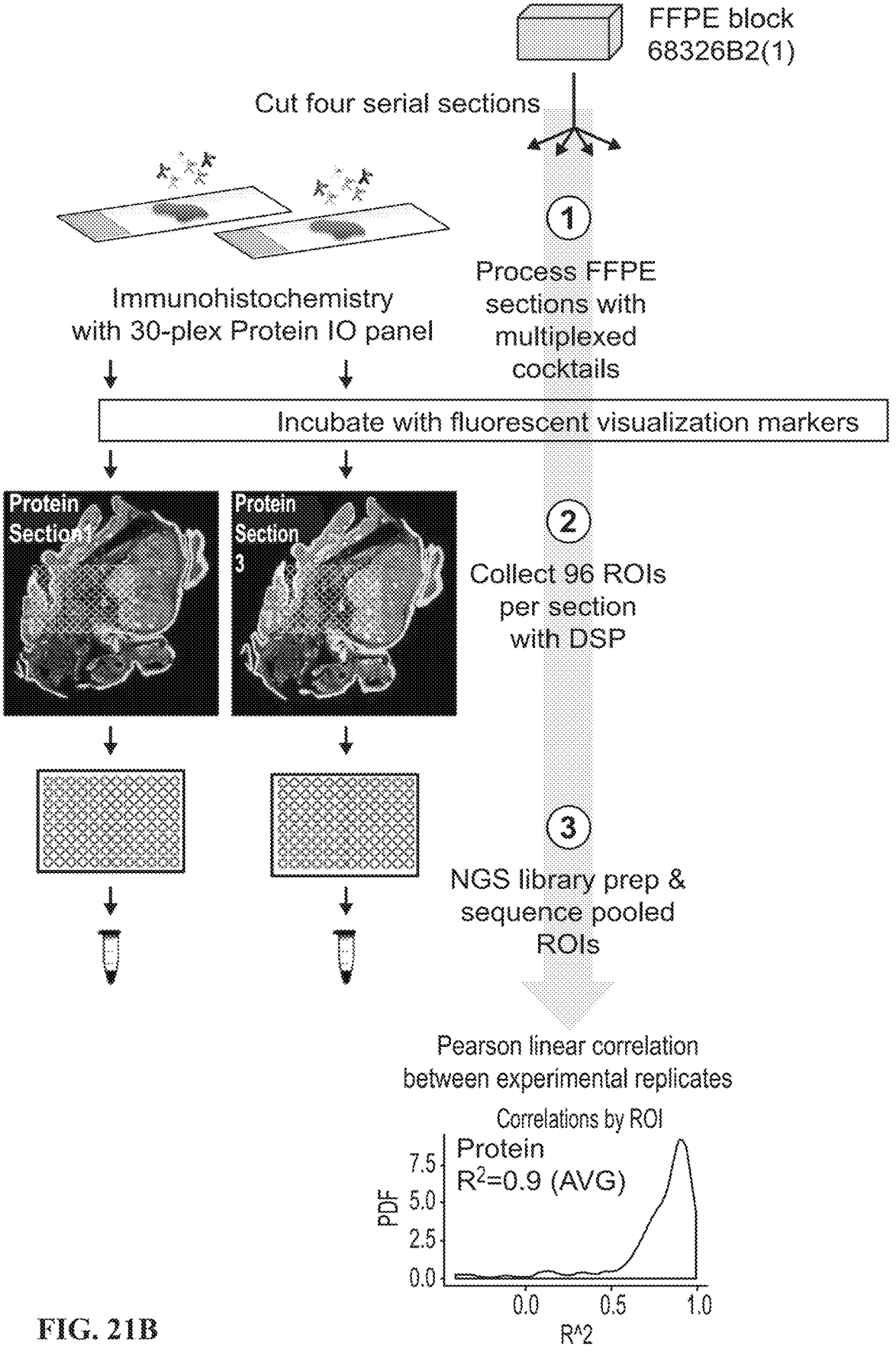

FFPE block
68326B2(1)
Cut four serial sections
1
Process FFPE sections with multiplexed cocktails
Immunohistochemistry with 30-plex Protein IO panel
Incubate with fluorescent visualization markers
Protein Section 1
Protein Section 3
2
Collect 96 ROIs per section with DSP
3
NGS library prep & sequence pooled ROIs
Pearson linear correlation between experimental replicates
Correlations by ROI
Protein
R2=0.9 (AVG)
PDF
7.5
5.0
2.5
0.0
0.0
0.5
1.0
R^2

FIG. 21B

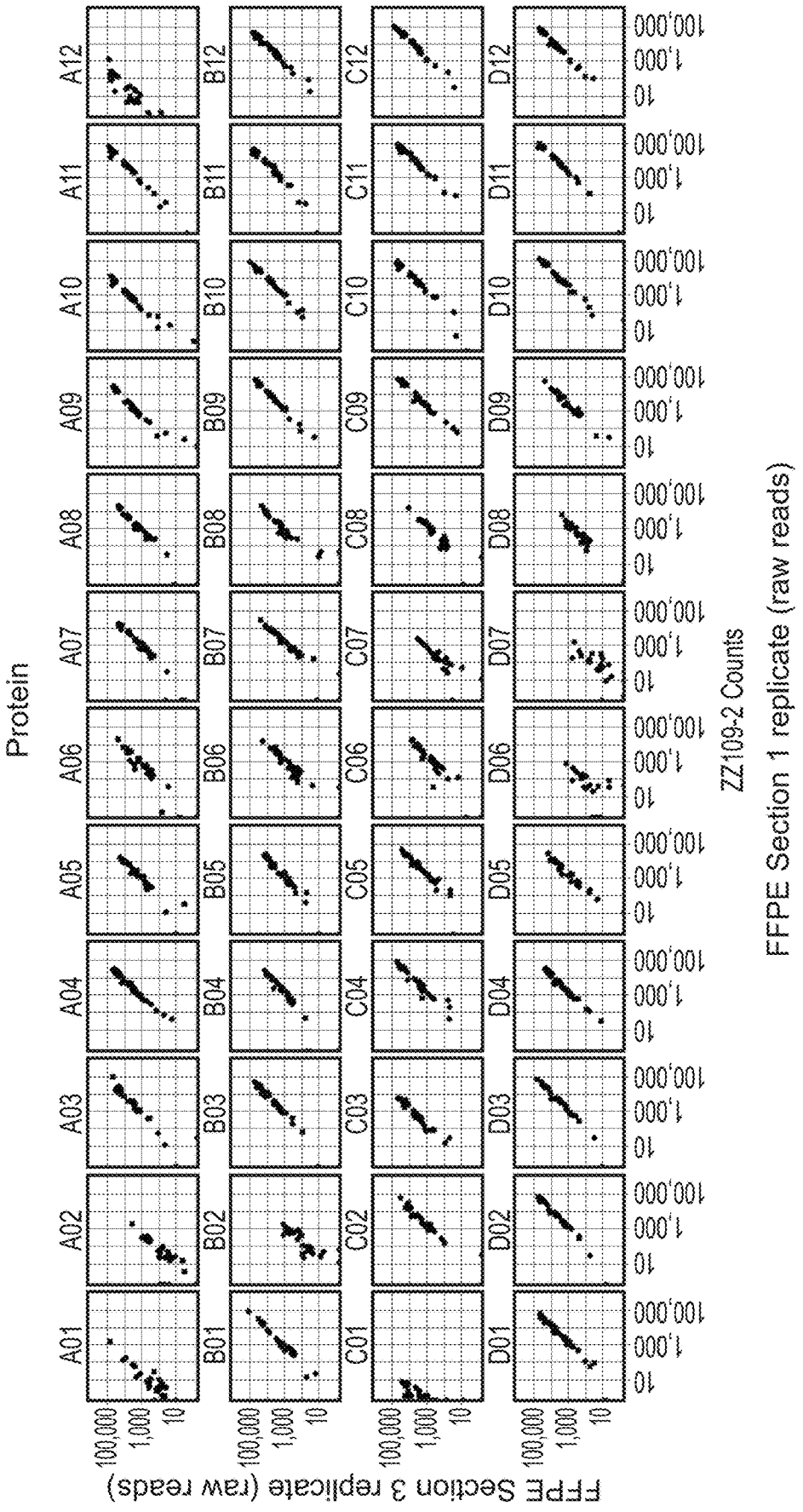

Protein
A01 A02 A03 A04 A05 A06 A07 A08 A09 A10 A11 A12
B01 B02 B03 B04 B05 B06 B07 B08 B09 B10 B11 B12
C01 C02 C03 C04 C05 C06 C07 C08 C09 C10 C11 C12
D01 D02 D03 D04 D05 D06 D07 D08 D09 D10 D11 D12
100,000
1,000
10
FFPE Section 3 replicate (raw reads)
ZZ109-2 Counts
FFPE Section 1 replicate (raw reads)

FIG. 21C

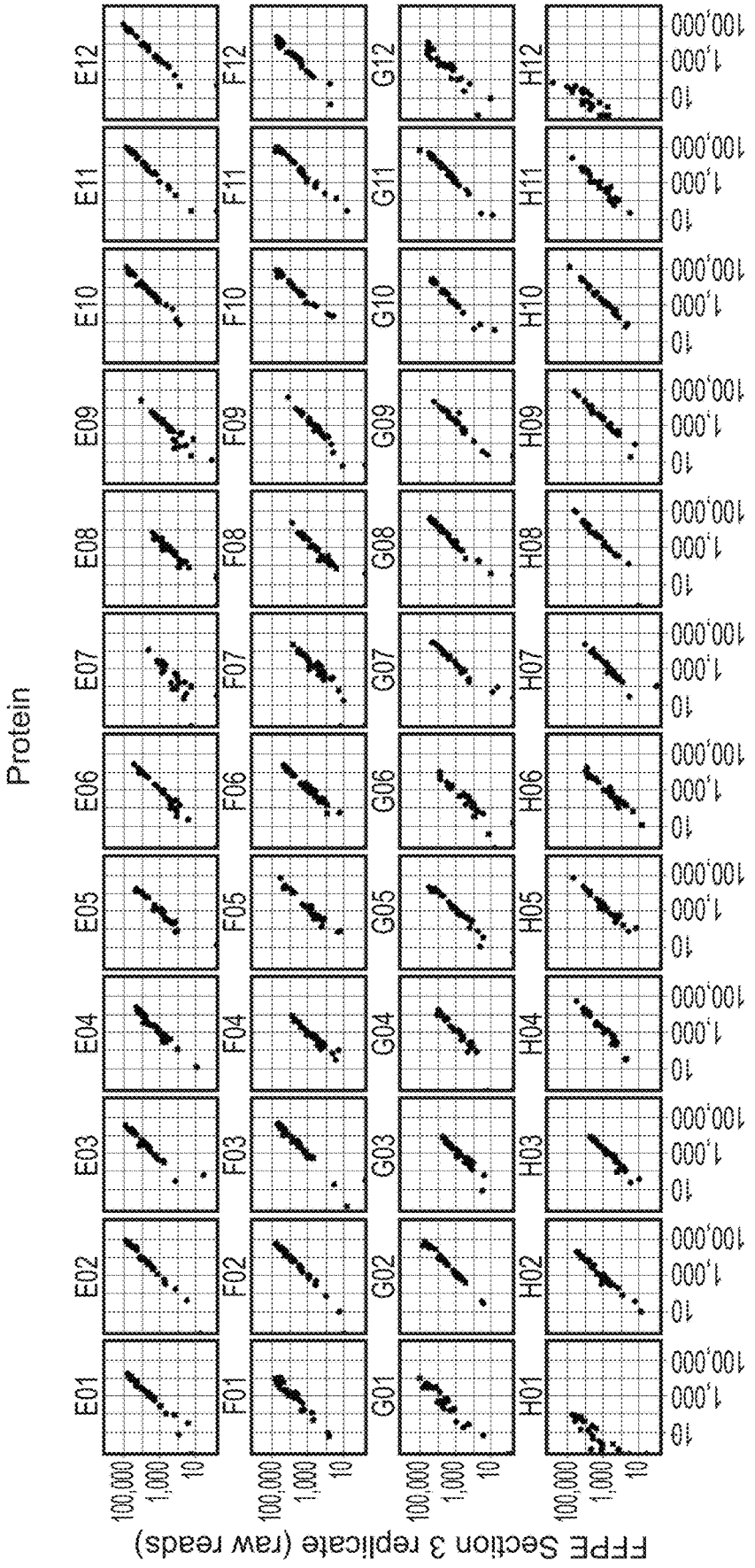

Protein
E01
E02
E03
E04
E05
E06
E07
E08
E09
E10
E11
E12
F01
F02
F03
F04
F05
F06
F07
F08
F09
F10
F11
F12
G01
G02
G03
G04
G05
G06
G07
G08
G09
G10
G11
G12
H01
H02
H03
H04
H05
H06
H07
H08
H09
H10
H11
H12
100,000
1,000
10
FFPE Section 3 replicate (raw reads)
ZZ109-2 Counts
FFPE Section 1 replicate (raw reads)

FIG. 21D

RNA Immuno-oncology Panel (20-plex)
CD3E
CD3G
CD4
CD20
CD40
CD45
CD74
CD79A
CTLA4
GAPDH
KRT13
PD1
PSA
RPS6
Negative probe 1
Negative probe 2
Negative probe 3
Negative probe 4
Negative probe 5
Negative probe 6

FIG. 22A

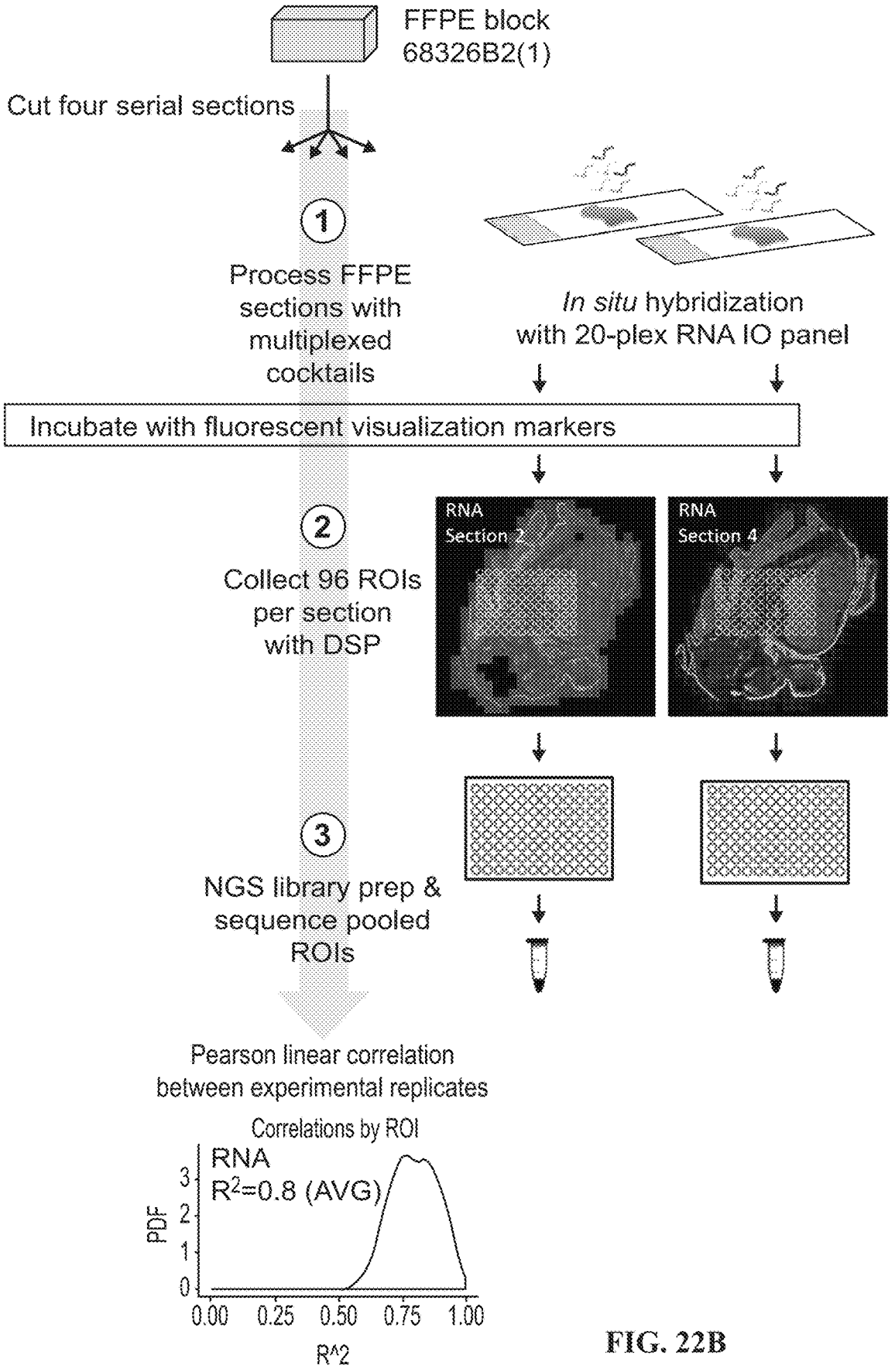

FFPE block
68326B2(1)
Cut four serial sections
1
Process FFPE sections with multiplexed cocktails
In situ hybridization with 20-plex RNA IO panel
Incubate with fluorescent visualization markers
2
Collect 96 ROIs per section with DSP
RNA Section 2
RNA Section 4
3
NGS library prep & sequence pooled ROIs
Pearson linear correlation between experimental replicates
Correlations by ROI
RNA
R2=0.8 (AVG)
PDF
0
1
2
3
0.00
0.25
0.50
0.75
1.00
R^2

FIG. 22B

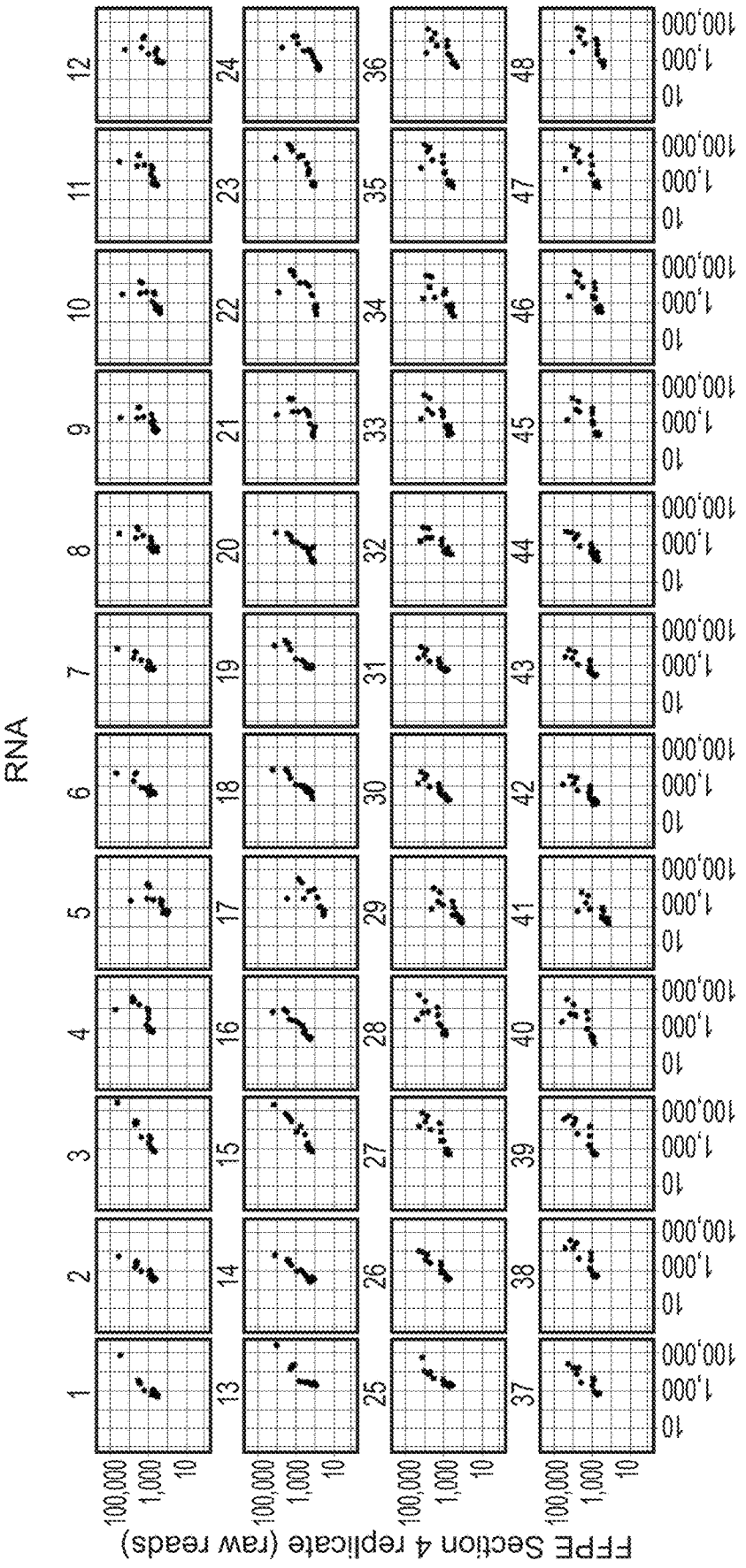

RNA
LD006 Counts
FFPE Section 2 replicate (raw reads)
FFPE Section 4 replicate (raw reads)
100,000
1,000
10

FIG. 22C

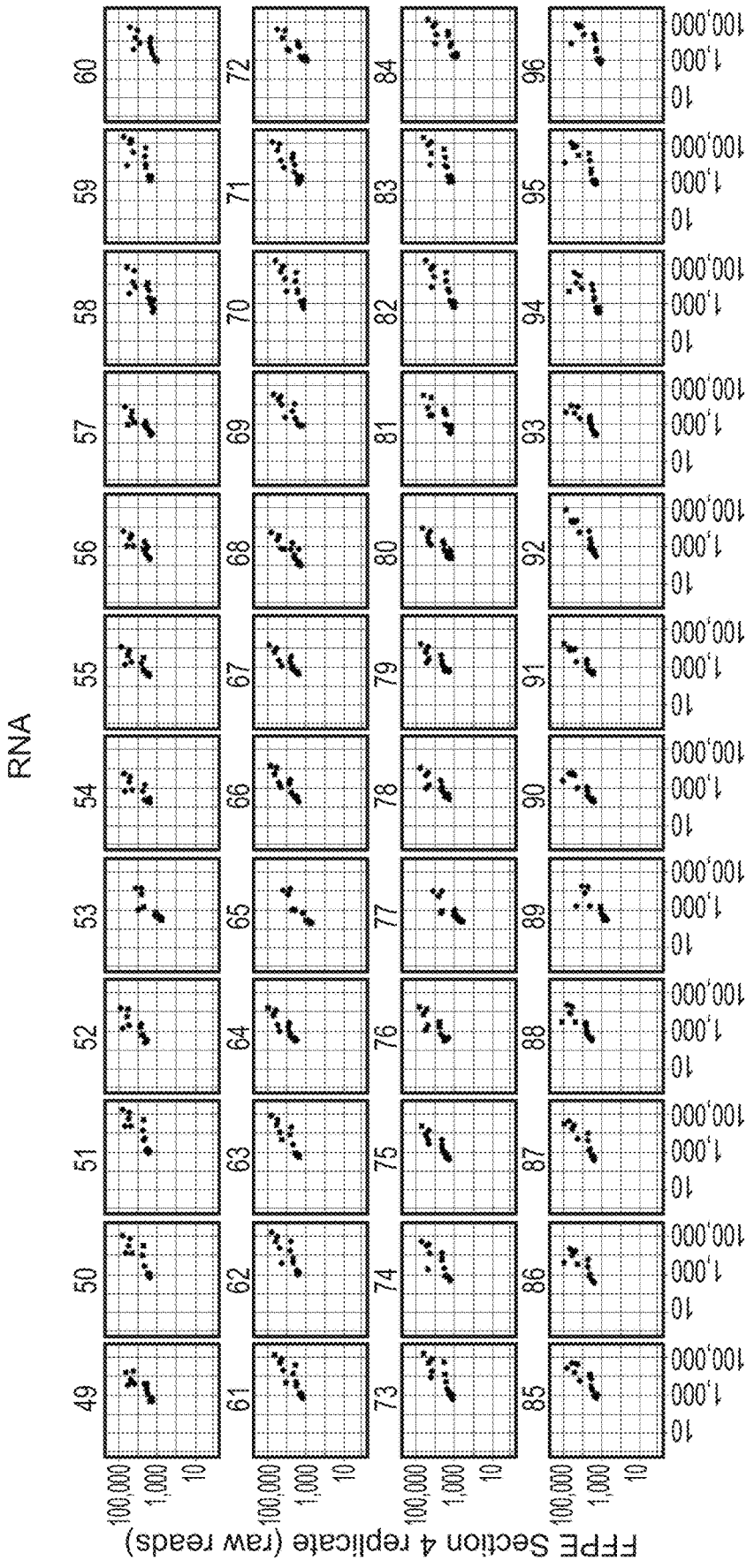

RNA
LD006 Counts
FFPE Section 2 replicate (raw reads)
FFPE Section 4 replicate (raw reads)
100,000
1,000
10
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96

FIG. 22D

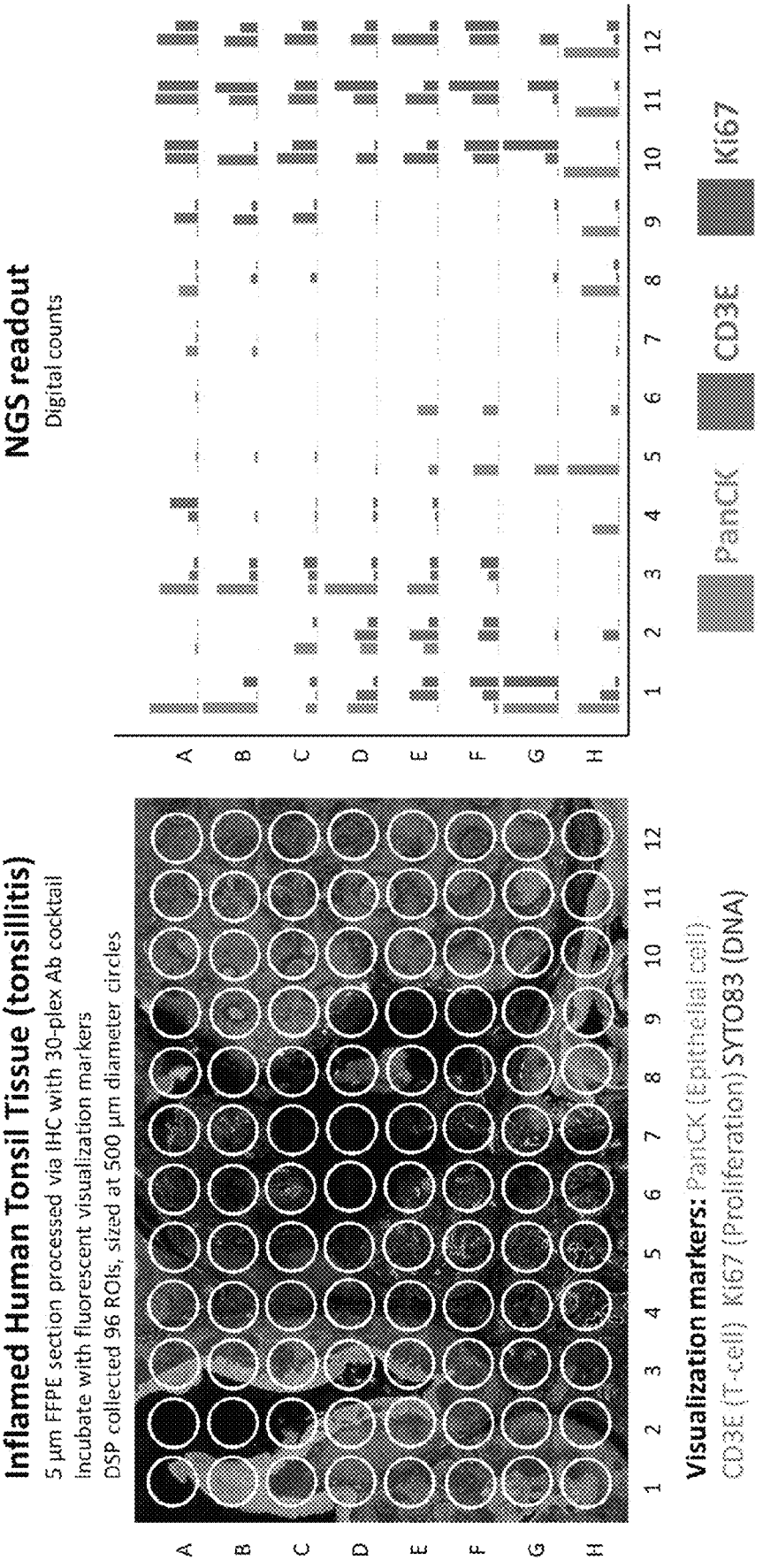

Inflamed Human Tonsil Tissue (tonsillitis)
5 µm FFPE section processed via IHC with 30-plex Ab cocktail
Incubate with fluorescent visualization markers
DSP collected 96 ROIs, sized at 500 µm diameter circles
Visualization markers: PanCK (Epithelial cell)
CD3E (T-cell) Ki67 (Proliferation) SYTO83 (DNA)
NGS readout
Digital counts
PanCK
CD3E
Ki67
A
B
C
D
E
F
G
H
1 2 3 4 5 6 7 8 9 10 11 12

FIG. 23

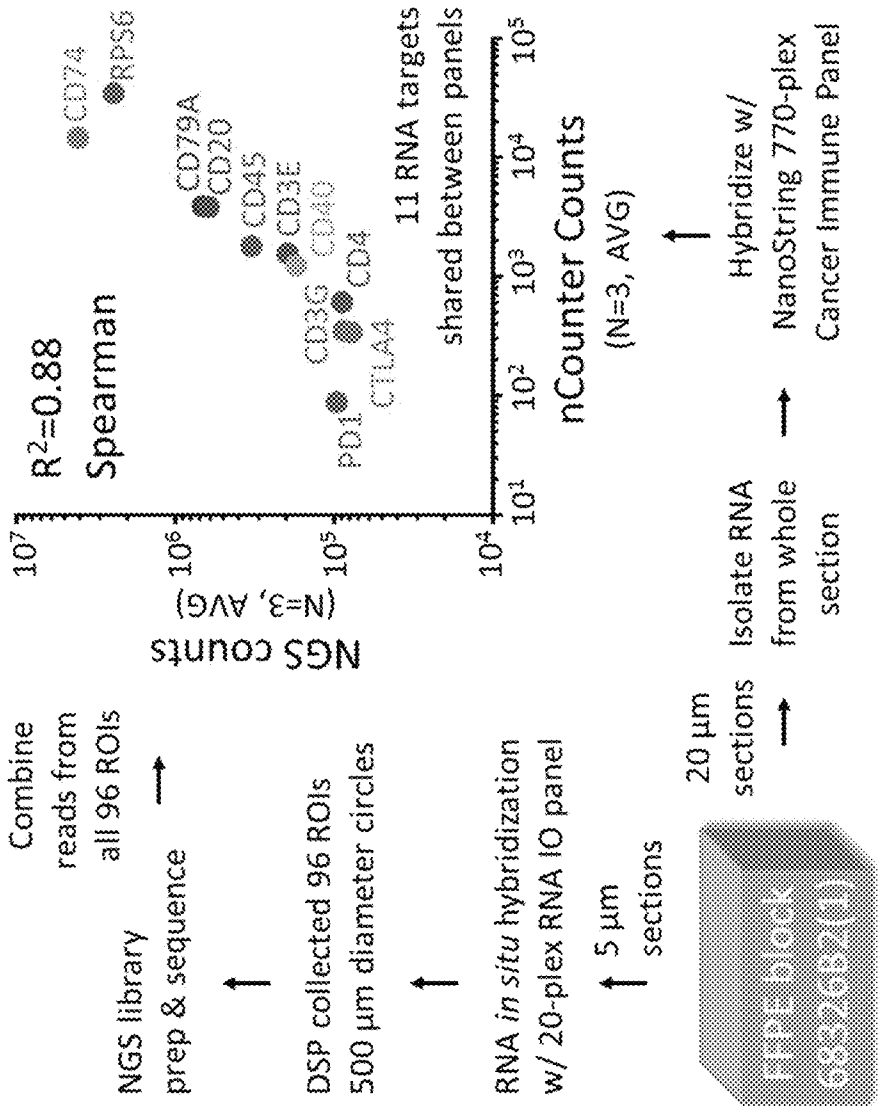

FFPE block
20 µm sections
Isolate RNA from whole section
Hybridize w/ NanoString 770-plex Cancer Immune Panel
5 µm sections
RNA in situ hybridization w/ 20-plex RNA IO panel
DSP collected 96 ROIs 500 µm diameter circles
NGS library prep & sequence
Combine reads from all 96 ROIs
NGS counts (N=3, AVG)
nCounter Counts (N=3, AVG)
R²=0.88
Spearman
11 RNA targets shared between panels
CD74
RPS6
CD79A
CD20
CD45
CD3E
CD40
CD4
CD36
CTLA4
PD1

FIG. 24

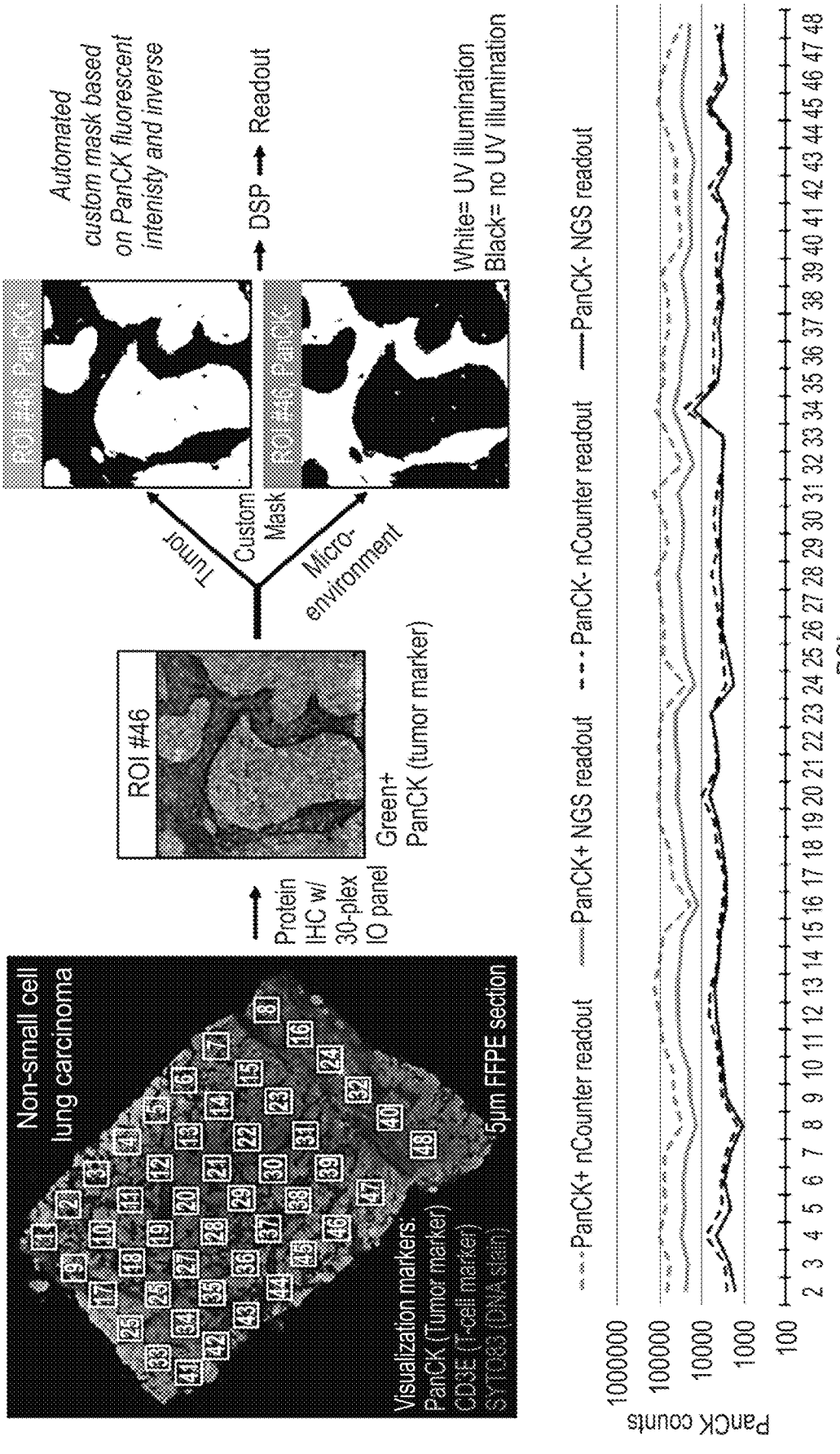
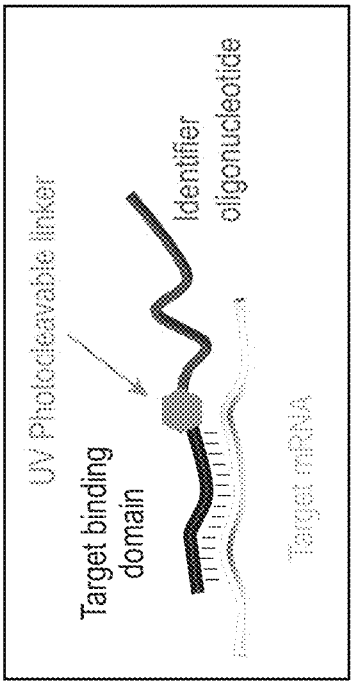

Non-small cell lung carcinoma
Visualization markers:
PanCK (Tumor marker)
CD3E (T-cell marker)
SYTO83 (DNA stain)
5um FFPE section
Protein IHC w/ 30-plex IO panel
ROI #46
Green+ PanCK (tumor marker)
Tumor
Custom Mask
Micro-environment
ROI PanCK
ROI PanCK
Automated custom mask based on PanCK fluorescent intenisty and inverse
DSP
Readout
White= UV illumination
Black= no UV illumination
PanCK+ nCounter readout
PanCK+ NGS readout
PanCK- nCounter readout
PanCK- NGS readout
PanCK counts
1000000
100000
10000
1000
100
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
ROI

FIG. 25


UV Photocleavable linker
Target binding domain
Identifier oligonucleotide
Target mRNA

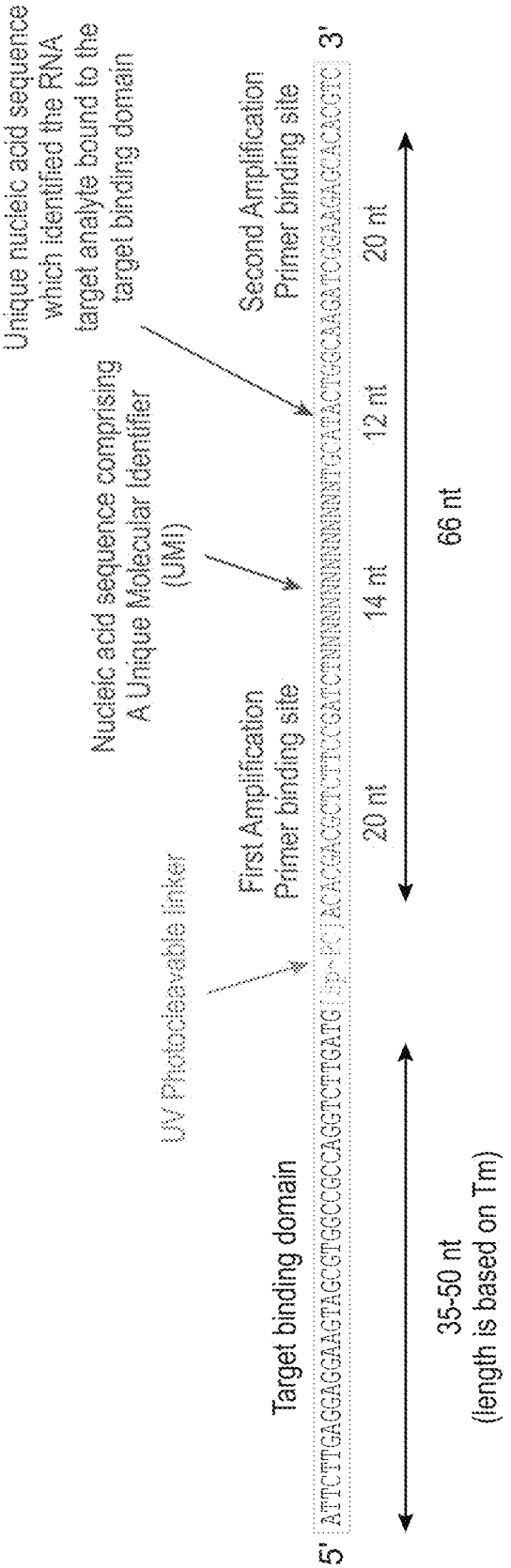

Unique nucleic acid sequence which identified the RNA target analyte bound to the target binding domain
Second Amplification Primer binding site
3'
Nucleic acid sequence comprising A Unique Molecular Identifier (UMI)
UV Photocleavable linker
First Amplification Primer binding site
20 nt
14 nt
12 nt
20 nt
66 nt
5'
Target binding domain
35-50 nt (length is based on Tm)

FIG. 26

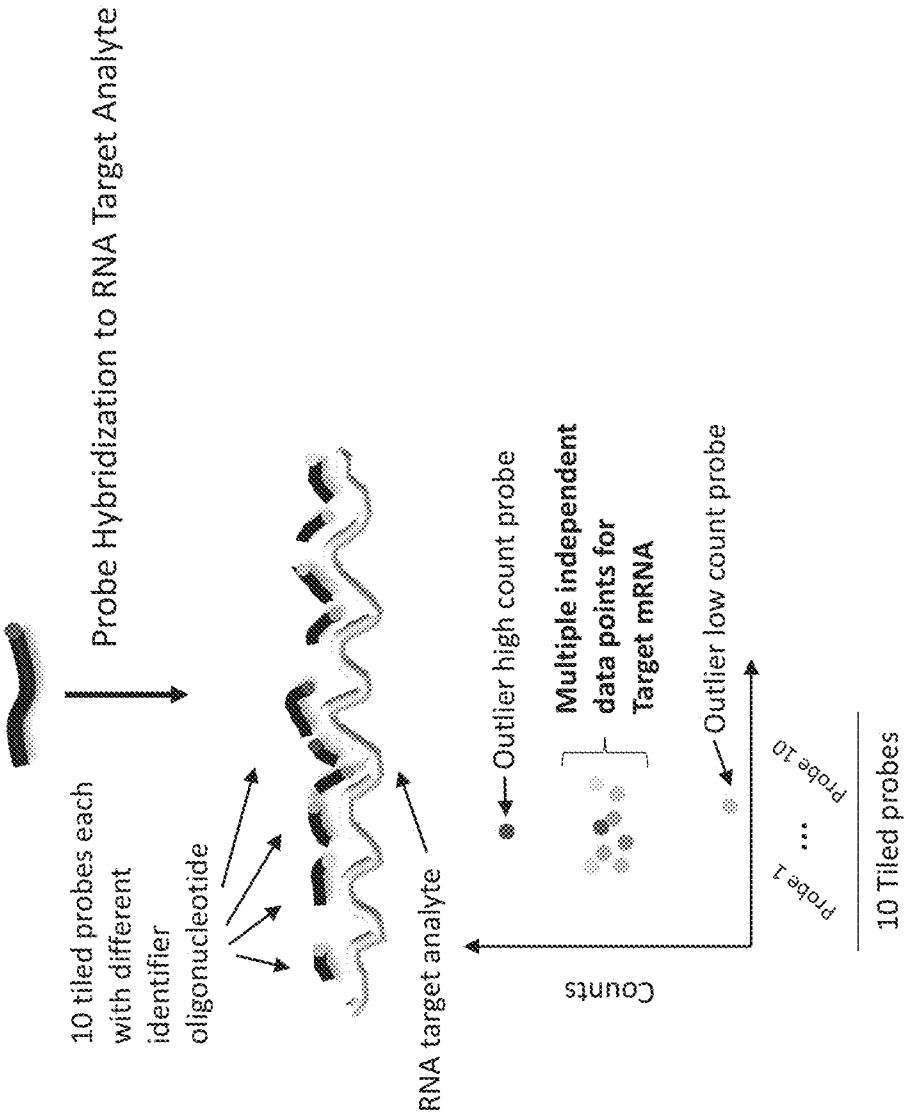

Probe Hybridization to RNA Target Analyte
10 tiled probes each with different identifier oligonucleotide
RNA target analyte
Outlier high count probe
Multiple independent data points for Target mRNA
Outlier low count probe
Counts
Probe 1
...
Probe 10
10 Tiled probes

FIG. 27

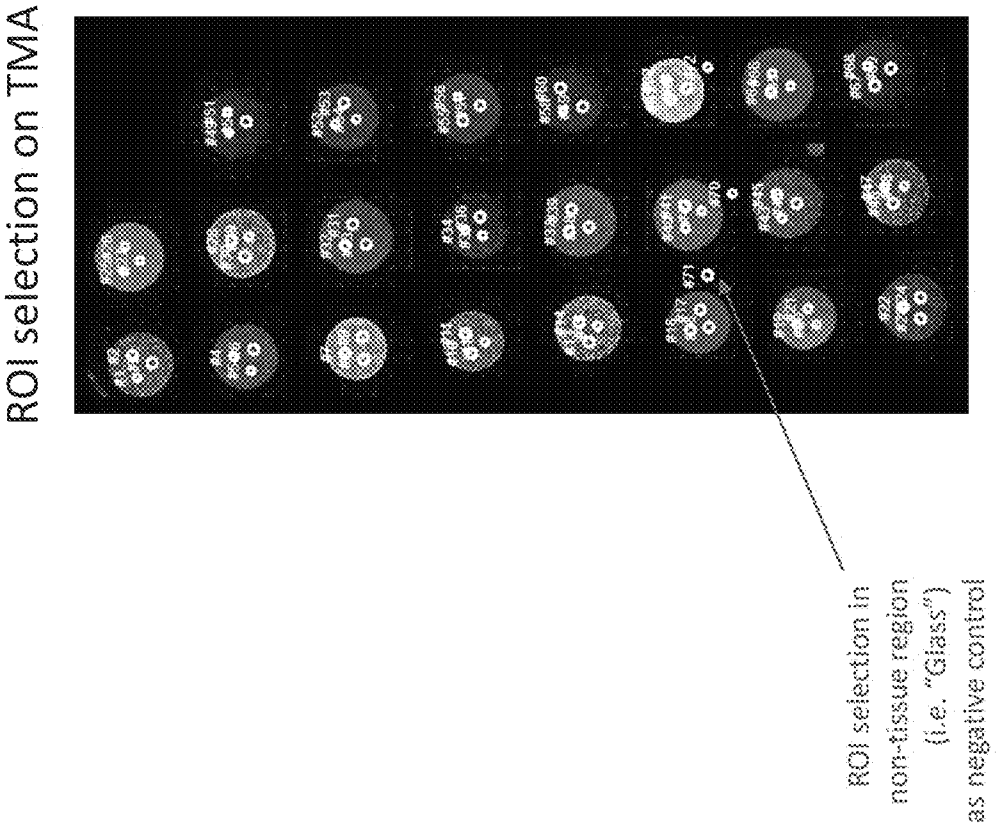

ROI selection on TMA
ROI selection in non-tissue region (i.e. "Glass") as negative control

FIG. 28

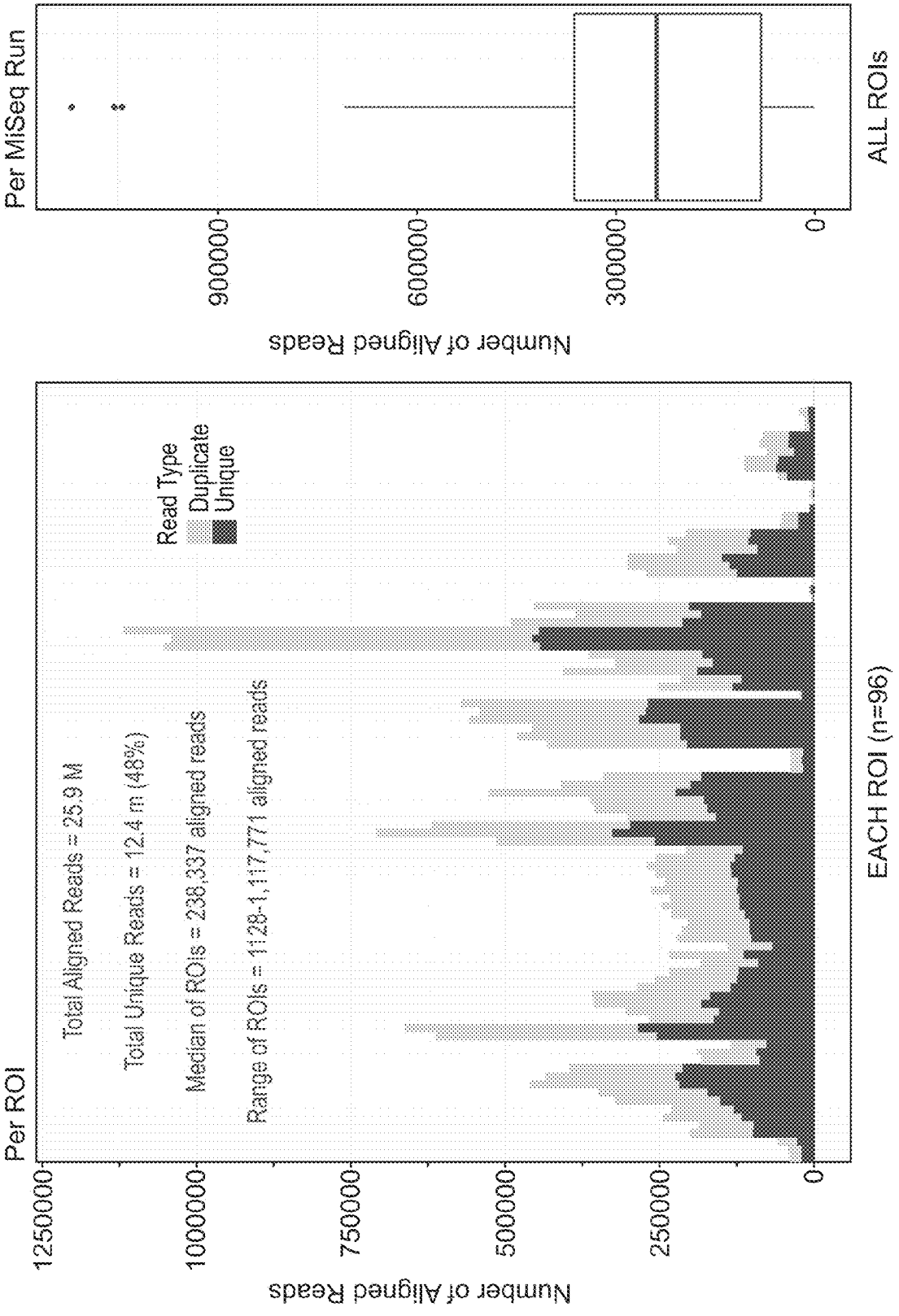

Per MiSeq Run
Number of Aligned Reads
900000
600000
300000
0
ALL ROIs
Per ROI
Total Aligned Reads = 25.9 M
Total Unique Reads = 12.4 m (48%)
Median of ROIs = 238,337 aligned reads
Range of ROIs = 1128-1,117,771 aligned reads
Read Type
Duplicate
Unique
1250000
1000000
750000
500000
250000
0
Number of Aligned Reads
EACH ROI (n=96)

FIG. 29

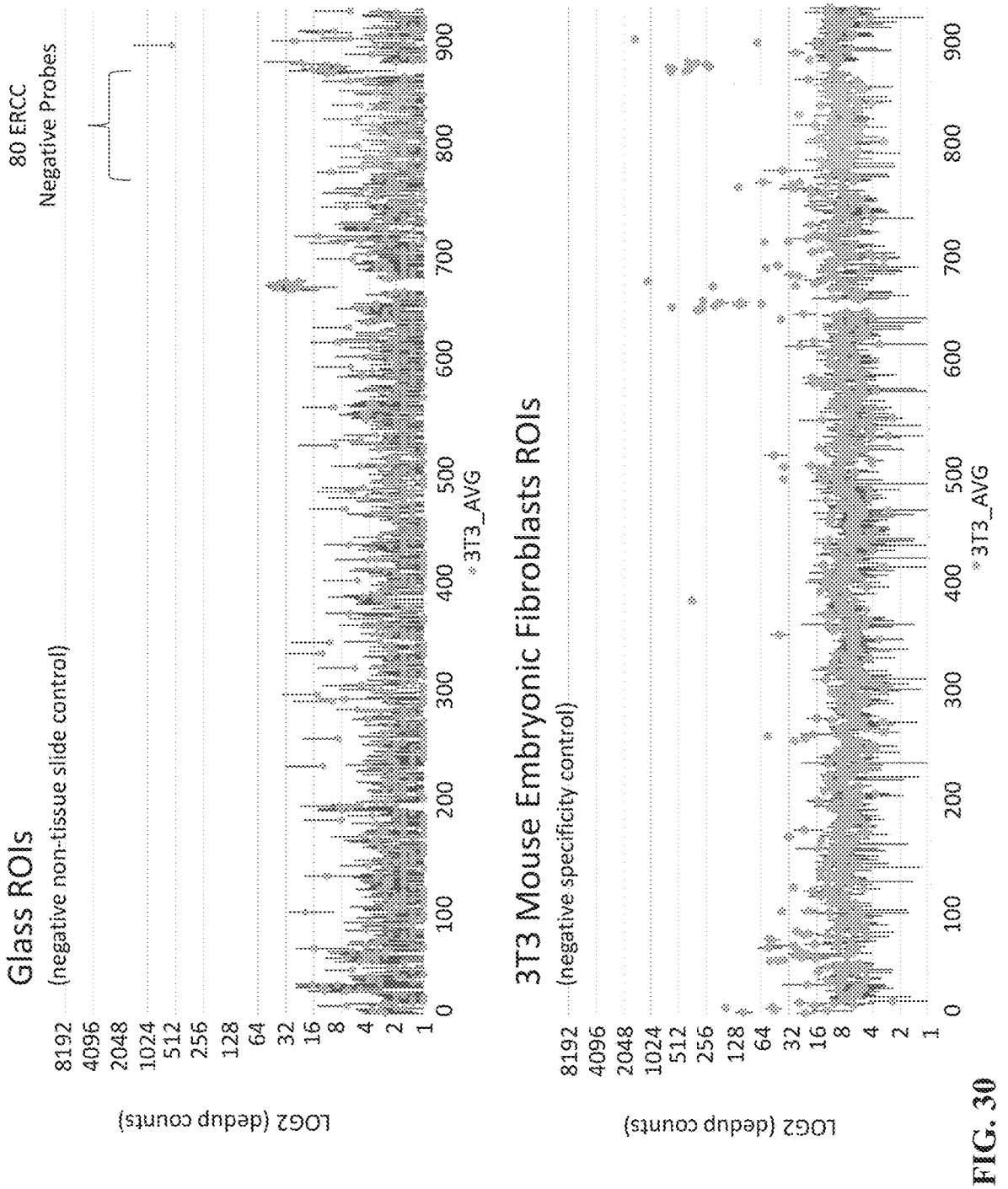

Glass ROIs
(negative non-tissue slide control)
80 ERCC
Negative Probes
LOG2 (dedup counts)
8192
4096
2048
1024
512
256
128
64
32
16
8
4
2
1
0
100
200
300
400
500
600
700
800
900
3T3_AVG
3T3 Mouse Embryonic Fibroblasts ROIs
(negative specificity control)
LOG2 (dedup counts)
3T3_AVG

FIG. 30

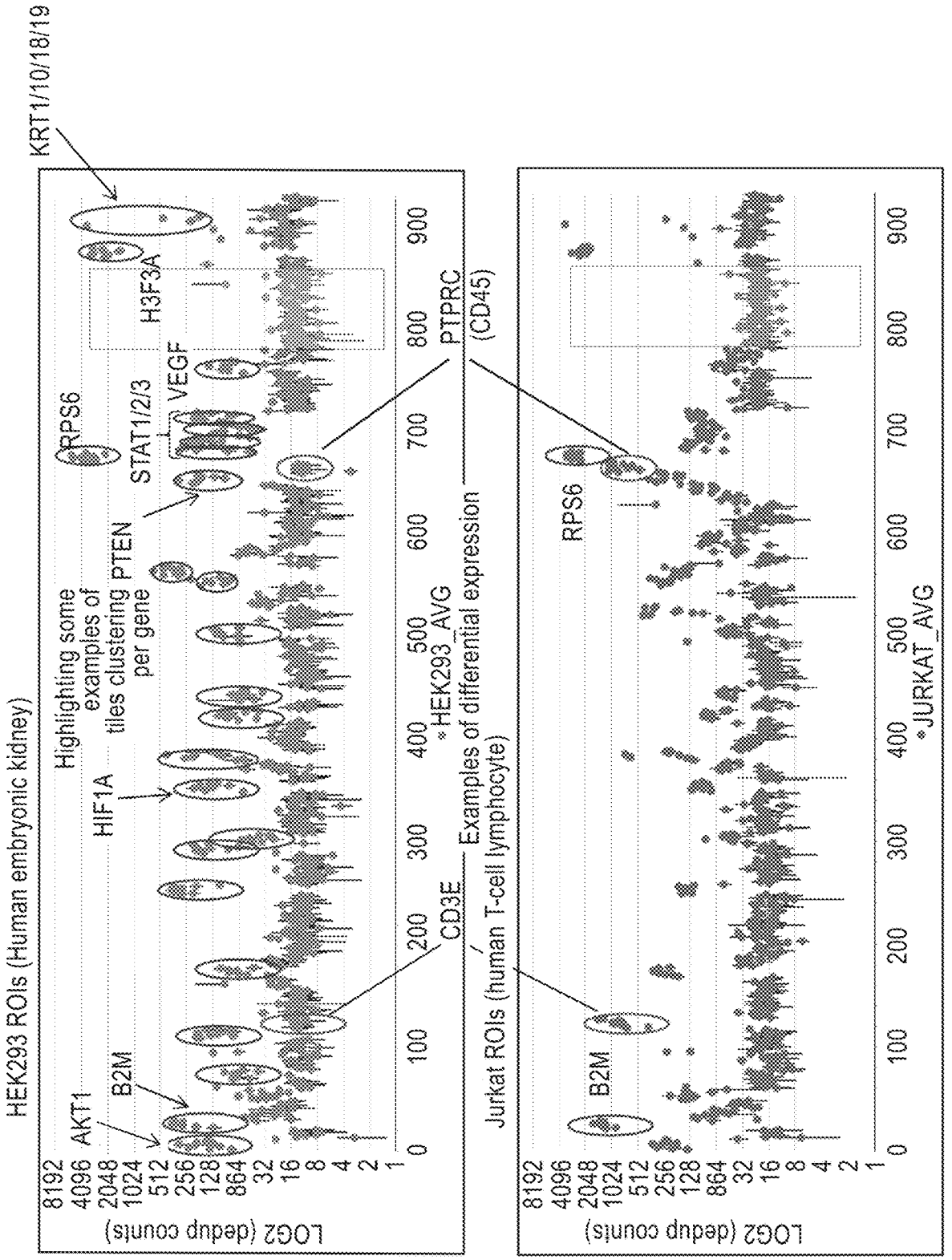

HEK293 ROIs (Human embryonic kidney)
KRT1/10/18/19
Highlighting some examples of tiles clustering per gene
RPS6
STAT1/2/3
VEGF
H3F3A
PTEN
HIF1A
AKT1
B2M
CD3E
PTPRC (CD45)
Examples of differential expression
Jurkat ROIs (human T-cell lymphocyte)
RPS6
B2M
LOG2 (dedup counts)
8192
4096
2048
1024
512
256
128
864
32
16
8
4
2
1
0
100
200
300
400
500
600
700
800
900
*HEK293_AVG
*JURKAT_AVG

FIG. 31

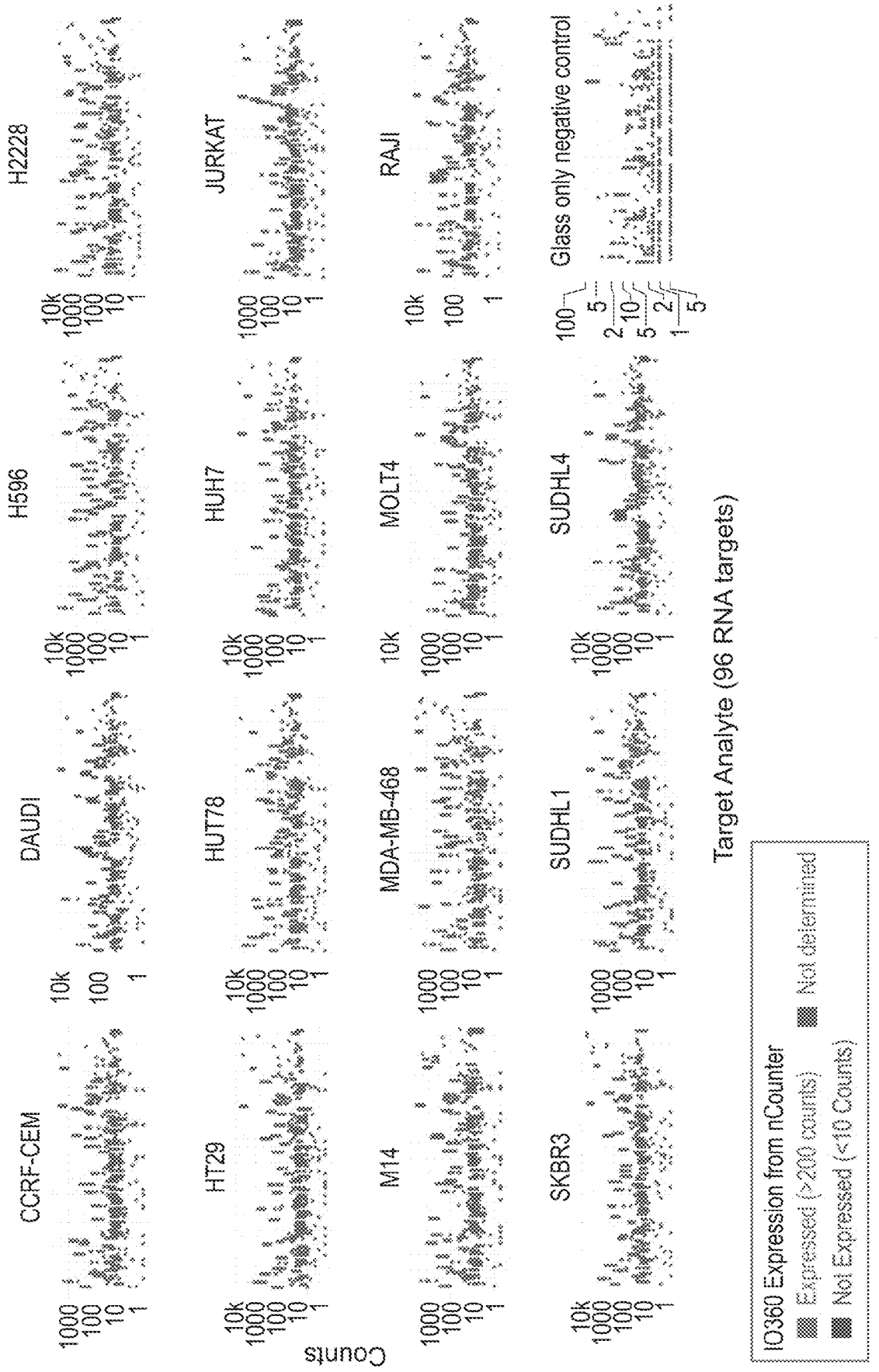

CCRF-CEM
DAUDI
H596
H2228
HT29
HUT78
HUH7
JURKAT
M14
MDA-MB-468
MOLT4
RAJI
SKBR3
SUDHL1
SUDHL4
Glass only negative control
Counts
Target Analyte (96 RNA targets)
IO360 Expression from nCounter
Expressed (>200 counts)
Not determined
Not Expressed (<10 Counts)

FIG. 32

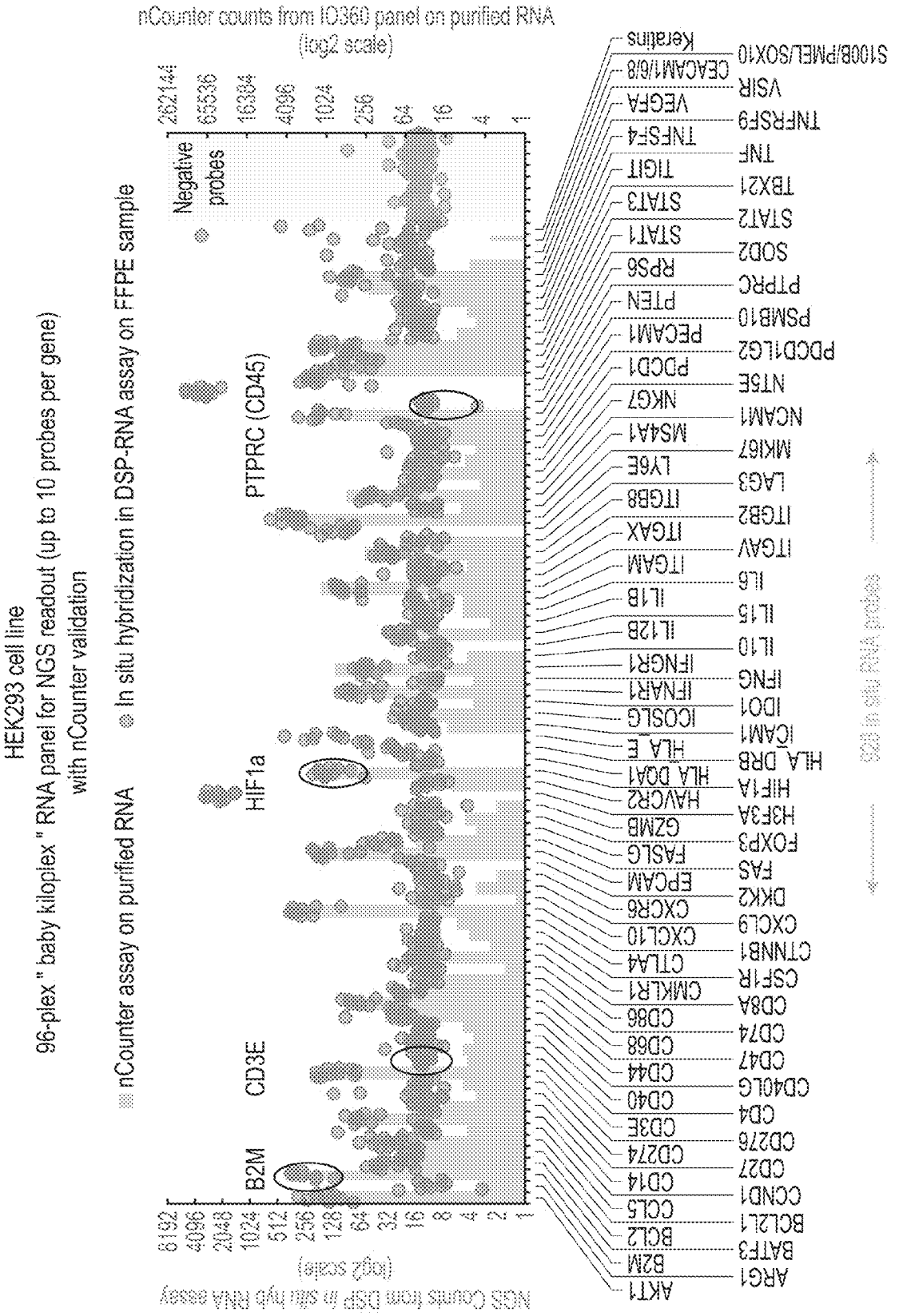

HEK293 cell line
96-plex " baby kioplex " RNA panel for NGS readout (up to 10 probes per gene)
with nCounter validation
nCounter assay on purified RNA
In situ hybridization in DSP-RNA assay on FFPE sample
nCounter counts from IO360 panel on purified RNA
(log2 scale)
NGS Counts from DSP in situ hyb RNA assay
(log2 scale)
Negative probes
B2M
CD3E
HIF1a
PTPRC (CD45)
AKT1
ARG1
BATF3
BCL2
BCL2L1
CCL5
CCND1
CD14
CD27
CD274
CD276
CD3E
CD4
CD40
CD40LG
CD44
CD47
CD74
CD68
CD86
CD8A
CMKLR1
CSF1R
CTLA4
CTNNB1
CXCL10
CXCL9
CXCR6
DKK2
EPCAM
FAS
FASLG
FOXP3
GZMB
H3F3A
HAVCR2
HIF1A
HLA_DOA1
HLA_DRB
HLA_E
ICAM1
ICOSLG
IDO1
IFNAR1
IFNG
IFNGR1
IL10
IL12B
IL15
IL1B
IL6
ITGAM
ITGAV
ITGAX
ITGB2
ITGB8
LAG3
LY6E
MKI67
MS4A1
NCAM1
NKG7
NT5E
PDCD1
PDCD1LG2
PECAM1
PSMB10
PTEN
PTPRC
RPS6
SOD2
STAT1
STAT2
STAT3
TBX21
TIGIT
TNF
TNFSF4
TNFRSF9
VEGFA
VSIR
CEACAM1/6/8
Keratins
S100B/PMEL/SOX10
GSK in situ RNA probes

FIG. 33

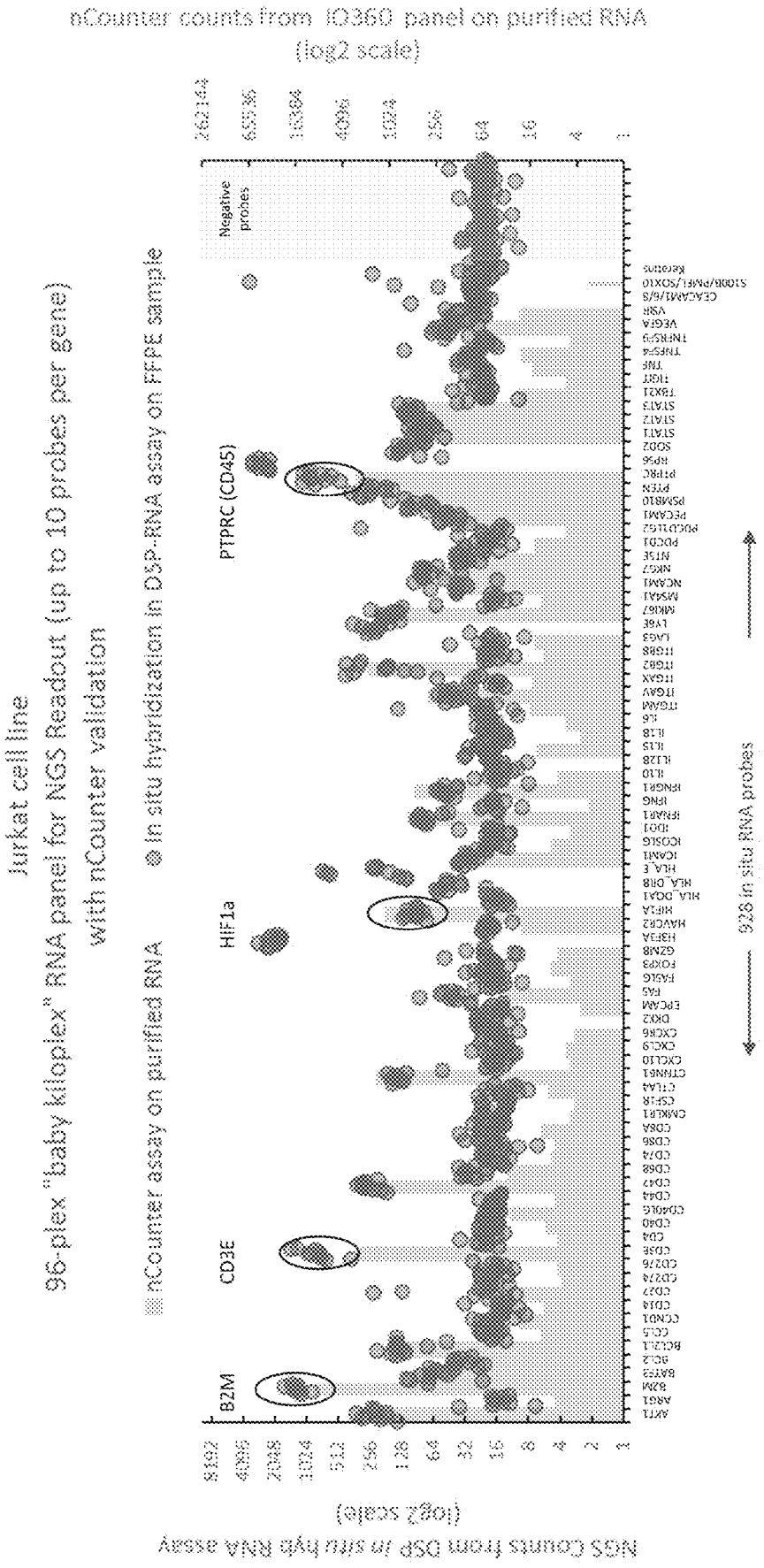

nCounter counts from IO360 panel on purified RNA
(log2 scale)
Jurkat cell line
96-plex "baby kiloplex" RNA panel for NGS Readout (up to 10 probes per gene)
with nCounter validation
nCounter assay on purified RNA
In situ hybridization in DSP-RNA assay on FFPE sample
Negative probes
B2M
CD3E
HIF1a
PTPRC (CD45)
926 in situ RNA probes
NGS Counts from DSP in situ hyb RNA assay
(log2 scale)

FIG. 34

CHEMICAL COMPOSITIONS AND USES THEREOF

This application is a continuation of U.S. patent application Ser. No. 16/272,487, filed Feb. 11, 2019. U.S. patent application Ser. No. 16/272,487 claims priority to, and the benefit of, U.S. Provisional Application No. 62/629,180, filed Feb. 12, 2018 and U.S. Provisional Application No. 62/771,212, filed Nov. 26, 2018. The contents of each of the aforementioned patent applications are incorporated herein by reference in their entireties.

BACKGROUND OF THE INVENTION

Sequence Listing

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Sep. 15, 2021, is named "NATE-037_CO1US_SeqList.txt" and is about 50,062 bytes in size.

BACKGROUND OF THE INVENTION

Standard immunohistochemical and in situ hybridization methods allow for simultaneous detection of, at most, six to ten protein or nucleic acid targets, with three to four targets being typical. There exists a need for probes, compositions, methods, and kits for simultaneous, multiplexed detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell. Furthermore, there is a need for such systems to be adaptable for use with existing sequencing technologies that are already available to a large number of end users.

SUMMARY OF THE INVENTION

The present disclosure relates to probes, compositions, methods, and kits for simultaneous, multiplexed, spatial detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell.

The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, a second amplification primer binding site and optionally, a constant nucleic acid sequence to minimize ligation bias; (5) amplifying the ligation product produced in step (4); and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises: a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, a second amplification primer binding site and optionally, a constant nucleic acid sequence to minimize ligation bias, and wherein at least one of the first or second amplification primer binding sites identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; (5) amplifying the ligation product produced in step (4); and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises: a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, a second amplification primer binding site and optionally, a constant nucleic acid sequence to minimize ligation bias; (5) amplifying the extension product produced in step (4) using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The nucleic acid adapter of step (4) can be a partially double-stranded nucleic acid molecule. A partially double-stranded nucleic acid adapter can comprise a double-stranded annealed region, a first single-stranded mismatched region and a second single-stranded mismatched region. The first single-stranded mismatched region and the second single stranded mismatched region can be present on opposing sides of the double-stranded annealed region.

The nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be present in the double-stranded annealed region of a partially double-stranded nucleic acid adapter.

The constant nucleic acid sequence to minimize ligation bias can be present in the double-stranded annealed region of a partially double-stranded nucleic acid adapter.

A unique molecular identifier can be present in at least one of the first or second single-stranded mismatched regions of a partially double-stranded nucleic acid adapter.

The first amplification primer binding site can be present in the first single-stranded mismatched region of a partially double-stranded nucleic acid adapter and the second amplification primer binding site can be present in the second single-stranded mismatched region of the same partially double-stranded nucleic acid adapter.

The methods of the present disclosure described in the preceding can further comprise prior to step (4), performing an end repair reaction. The methods can also further comprise prior to step (4), performing a tailing reaction to attach a single nucleotide overhang to the 3' ends of the identifier oligonucleotide. The methods can further comprise, prior to step (4), performing an end repair reaction and a tailing reaction to attach a single nucleotide overhang to the 3' ends of the identifier oligonucleotide. The tailing reaction and the end repair reaction can be performed sequentially or concurrently.

The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a first amplification primer binding site, and a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier, a second amplification primer binding site and optionally, a constant nucleic acid sequence to minimize ligation bias; (5) amplifying the ligation product produced in step (4); and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises: a first amplification primer binding site and a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises a nucleic acid sequence comprising a unique molecular identifier, a second amplification primer binding site, and optionally, a constant nucleic acid sequence to minimize ligation bias; (5) amplifying the extension product produced in step (4) using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The nucleic acid adapter of step (4) can be a partially double-stranded nucleic acid molecule. A partially double-stranded nucleic acid adapter can comprise a double-stranded annealed region and a single-stranded mismatched region.

The nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be present in the double-stranded annealed region of a partially double-stranded nucleic acid adapter.

The constant nucleic acid sequence to minimize ligation bias can be present in the double-stranded annealed region of a partially double-stranded nucleic acid adapter. The constant nucleic acid sequence can also comprise a cleavable moiety. The cleavable moiety can be an enzymatically cleavable moiety. The enzymatically cleavable moiety can be a USER sequence.

A unique molecular identifier can be present in the single-stranded mismatched region of a partially double-stranded nucleic acid adapter.

The second amplification primer binding site can be present in the single-stranded mismatched region of a partially double-stranded nucleic acid adapter.

The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a first amplification primer binding site, and a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a single stranded nucleic acid template, wherein the nucleic acid template comprises a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier, a nucleic acid sequence complementary to a second amplification primer binding site and optionally, an affinity molecule; (5) extending the identifier oligonucleotide of step (4) to form an extension product complementary to the single stranded nucleic acid template, wherein the extension product comprises the identifier oligonucleotide, the nucleic acid sequence complementary to the unique molecular identifier, and the second amplification primer binding site; (6) amplifying the extension product produced in step (5) using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; (7) Identifying the released identifier oligonucleotide by sequencing the amplified products pro-

duced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a first amplification primer binding site and a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a single stranded nucleic acid template, wherein the nucleic acid template comprises a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier, a nucleic acid sequence complementary to a second amplification primer binding site and optionally, an affinity molecule; (5) extending the identifier oligonucleotide of step (4) to form an extension product complementary to the single stranded nucleic acid template, wherein the extension product comprises the identifier oligonucleotide, the nucleic acid sequence complementary to the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, the nucleic acid sequence complementary to the unique molecular identifier and the second amplification primer binding site; (6) amplifying the extension product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The single stranded nucleic acid template can further comprise an affinity molecule. In aspects in which the single stranded nucleic acid template comprises an affinity molecule, the methods of the present disclosure described in the preceding can further comprise an affinity purification step between steps (4) and (5).

The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a first amplification primer binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier and a second amplification primer binding site, and wherein the first the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide and a first amplification primer binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide and a second amplification primer binding site, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a unique molecular identifier, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a first amplification primer binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier and a second amplification primer binding site, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first

and the second nucleic acid probes are not adjacent and are not overlapping; (5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide and a first amplification primer binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide and a second amplification primer binding site, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a unique molecular identifier, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping; (5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

In aspects in which the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released is located on the first nucleic acid probe, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be located 5' to the first amplification primer binding site.

In aspects in which a unique molecular identifier is located on the second nucleic acid probe, the unique molecular identifier can be located 3' to the second amplification primer binding site.

In aspects in which the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a unique molecular identifier are present in the first nucleic acid probe, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a unique molecular identifier can be located 5' to the first amplification primer binding site.

In aspects in which the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a unique molecular identifier are present in the second nucleic acid probe, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the unique molecular identifier can be located 3' to the second amplification primer binding site.

In aspects in which a unique molecular identifier is present in the first nucleic acid probe and the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released is present in the second nucleic acid probe, the unique molecular identifier can be located 5' to the first amplification primer binding site and the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be located 3' to the second amplification binding site.

The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a first amplification primer binding site, a nucleic acid sequence comprising a first unique molecular identifier and a first flow cell binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a second unique molecular identifier, a nucleic acid sequence comprising a third unique molecular identifier and a second flow cell binding site, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a first ampli-

fication primer binding site, a nucleic acid sequence comprising a first unique molecular identifier and a first flow cell binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a second unique molecular identifier and a second flow cell binding site, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a third unique molecular identifier, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a first amplification primer binding site, a nucleic acid sequence comprising a first unique molecular identifier and a first flow cell binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to the identifier oligonucleotide, a nucleic acid sequence comprising a second unique molecular identifier, a nucleic acid sequence comprising a third unique molecular identifier and a second flow cell binding site, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping; (5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to the identifier oligonucleotide, a first amplification primer binding site, a nucleic acid sequence comprising a first unique molecular identifier and a first flow cell binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to the identifier oligonucleotide, a nucleic acid sequence comprising a second unique molecular identifier and a second flow cell binding site, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a third unique molecular identifier, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping; (5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

In aspects in which the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the first unique molecular identifier are present in the first nucleic acid probe, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the first unique molecular identifier can be located 5' to the first flow cell binding site.

In aspects in which the second and the third unique molecular identifiers are present in the second nucleic acid probe, the second and the third unique molecular identifiers can be located 3' to the second flow cell binding site.

In some aspects, the first unique molecular identifier can be present in the first nucleic acid probe and can be located 5' to the first flow cell binding site. In other aspects, the second unique molecular identifier can be present in the second nucleic acid probe and can be located 3' to the second flow cell binding site.

In some aspects, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the third unique molecular identifier can be present in the first nucleic acid probe and can be located 5' to the first flow cell binding site.

In some aspects, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the third unique molecular identifier can be present in the second nucleic acid probe and can be located 3' to the second flow cell binding site.

In some aspect, the third unique molecular identifier can be present in the first nucleic acid probe and can be located 5' to the first flow cell binding site. In this same aspect, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be present in the second nucleic acid probe and can be located 3' to the second flow cell binding site.

The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain, a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, and a second amplification primer binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) amplifying the released identifier oligonucleotide using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; (5) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain, a first amplification primer binding site and a second amplification primer binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) amplifying the released identifier oligonucleotide using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein at least one of the amplification primers comprises a nucleic acid sequence comprising a unique molecular identifier; and (5) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain and a capture probe binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a capture probe, wherein the capture probe comprises an affinity molecule and a region complementary to the capture probe binding site; and (5) Identifying the released identifier oligonucleotide by sequencing the amplified hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain, a capture probe binding site and a multiplexing probe binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a capture probe and a multiplexing probe, wherein the capture probe comprises an affinity molecule and a region complementary to the capture probe binding site, and wherein the multiplexing probe comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a region complementary to the multiplexing probe binding site; and (5) Identifying the released identifier oligonucleotide by sequencing the hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises: a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, and wherein the second nucleic acid probe comprises: a nucleic acid complementary to a portion of the identifier oligonucleotide, and a second amplification primer binding site, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) ligating the hybridized first and second nucleic acid probes together; (6) amplifying the ligation product produced in step (5); and (7) identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises: a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain, a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, and a second ampli-

fication primer binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) amplifying the collected identifier oligonucleotide; (5) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

A In all methods of the present disclosure, the ligation process can be a nick ligation process. The nick ligation process can be a nick repair process.

In all methods of the present disclosure, the sequencing can be an enzyme free sequencing method.

In all methods of the present disclosure, the identifier oligonucleotide can be double-stranded. In aspects in which the identifier oligonucleotide is double-stranded, at least one of the two strands of the identifier oligonucleotide can comprise at least two separate nucleic acid molecules. Alternatively, at least one 3' end of an identifier oligonucleotide can comprise a single nucleotide overhang.

In all methods of the present disclosure, the identifier oligonucleotide can be single-stranded.

In all methods of the present disclosure, the unique nucleic acid sequence which identifies the target analyte bound to a target binding domain can comprise between about 5 nucleotides and about 40 nucleotides preferably about 35 nucleotides, preferably still about 10 nucleotides.

In all methods of the present disclosure, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can comprise between about 6 nucleotides and about 15 nucleotides, preferably about 12 nucleotides, preferably still about 10 nucleotides.

In all methods of the present disclosure, at least one of a first nucleic acid probe or a second nucleic acid probe can comprise an affinity molecule. For example, at least one of a first nucleic acid probe or a second nucleic acid probe can comprise a biotin.

In all methods of the present disclosure, an amplification primer binding site can comprise between about 18 nucleotides and about 40 nucleotides, preferably about 32 nucleotides, preferably still about 25 nucleotides. An amplification primer binding site can comprise an i7 sequence, wherein the i7 sequence comprises the sequence set forth in SEQ ID NO: 1. An amplification primer binding site can comprise an i5 sequence, wherein the i5 sequence comprises the sequence set forth in SEQ ID NO: 2.

In all methods of the present disclosure, an amplification primer can comprise a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing. An amplification primer can comprise a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3. An amplification primer can comprise a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

In all methods of the present disclosure, a flow cell binding site can comprise a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing. A flow cell binding site can comprise a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3. A flow cell binding site can comprise a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

In all methods of the present invention, at least one of the amplification primers can comprise an affinity molecule. For example, at least one of the amplification primers cam comprise a biotin.

In all methods of the present disclosure, amplification can comprise performing PCR. Performing PCR can comprise an amplification primer.

An amplification primer can comprise a flow cell binding site. An amplification primer can comprise a nucleic sequence which identifies the specific location of the tissue sample from which an identifier oligonucleotide was released. An amplification primer can comprise a nucleic acid sequence complementary to an amplification primer binding site.

Any of the above aspects can be combined with any other aspect.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. In the Specification, the singular forms also include the plural unless the context clearly dictates otherwise; as examples, the terms “a,” “an,” and “the” are understood to be singular or plural and the term “or” is understood to be inclusive. By way of example, “an element” means one or more element. Throughout the specification the word “comprising,” or variations such as “comprises” or “comprising,” will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from the context, all numerical values provided herein are modified by the term “about.”

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. The references cited herein are not admitted to be prior art to the claimed invention. In the case of conflict, the present Specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting. Other features and advantages of the disclosure will be apparent from the following detailed description and claim.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

The above and further features will be more clearly appreciated from the following detailed description when taken in conjunction with the accompanying drawings.

FIG. **1** is a schematic of a two-ended adapter ligation method of the present disclosure.

FIG. **2** is a schematic of a one-ended adapter ligation method of the present disclosure.

FIG. **3** is a schematic of a template-primer extension method of the present disclosure.

FIG. **4** is a schematic of a template-extended identifier oligonucleotide of the present disclosure.

FIG. **5** is a schematic of a short probe hybridization method of the present disclosure.

FIG. **6** is a schematic of a short probe hybridization method of the present disclosure.

FIG. **7** is a schematic of a short probe hybridization method of the present disclosure.

FIG. **8** is a schematic of a long probe hybridization method of the present disclosure.

FIG. **9** is a schematic of a long probe hybridization method of the present disclosure.

FIG. **10** is a schematic of a long probe hybridization method of the present disclosure.

FIG. **11** is a schematic of a direct-PCR method of the present disclosure.

FIG. **12** is a schematic of an enzyme free method of the present disclosure.

FIG. **13** is a schematic of a multiplexed enzyme free method of the present disclosure.

FIG. **14** is a schematic of a probe of the present disclosure indirectly binding to a target nucleic acid.

FIG. **15** is a schematic of an identifier oligonucleotide-short nucleic acid probe complex of the present disclosure.

FIG. **16** is a schematic of a short probe hybridization method of the present disclosure.

FIG. **17** is a schematic of an identifier oligonucleotide-short nucleic acid probe complex of the present disclosure.

FIG. **18** is a schematic of a short probe hybridization method of the present disclosure.

FIG. **19** is a schematic of a direct-PCR method of the present disclosure.

FIG. **20** is a schematic overview of the methods of the present disclosure.

FIG. **21**A, FIG. **21**B, FIG. **21**C and FIG. **21**D show the spatial detection of protein target analytes using the methods of the present disclosure.

FIG. **22**A, FIG. **22**B, FIG. **22**C and FIG. **22**D show the spatial detection of RNA target analytes using the methods of the present disclosure.

FIG. **23** shows the spatial detection of protein target analytes using the methods of the present disclosure.

FIG. **24** shows the spatial detection of RNA target analytes using the methods of the present disclosure.

FIG. **25** shows the spatial detection of protein target analytes using the methods of the present disclosure.

FIG. **26** is a schematic of a probe of the present disclosure. The nucleic acid sequence shown in FIG. **26** corresponds to SEQ ID NO: 175.

FIG. **27** shows the use of probe tiling in the methods of the present disclosure.

FIG. **28** shows the regions of interest selected on a tissue microarray.

FIG. **29** is a series of graphs showing the read depth achieved using the methods of the present disclosure.

FIG. **30** is a series of graphs showing the spatial detection of RNA target analytes in negative control samples using the methods of the present disclosure.

FIG. **31** is a series of graphs showing the spatial detection of RNA target analytes in a HEK293 sample (top panel) and a Jurkat cell sample (bottom panel) using the methods of the present disclosure.

FIG. **32** is a series of graphs showing the spatial detection of RNA target analytes in sixteen FFPE samples using the methods of the present disclosure.

FIG. **33** is a graph showing the spatial detection of RNA target analytes in a HEK293 sample using the methods of the present disclosure.

FIG. **34** is a graph showing the spatial detection of RNA target analytes in a Jurkat cell sample using the methods of the present disclosure.

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure is based in part on probes, compositions, methods, and kits for simultaneous, multiplexed spatial detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell using existing sequencing methods.

The present disclosure provides a comparison of the identity and abundance of target proteins and/or target nucleic acids present in a first region of interest (e.g., tissue type, a cell (including normal and abnormal cells), and a subcellular structure within a cell) and the identity and abundance of target proteins and/or target nucleic acids present in a second region of interest. There is no pre-defined upper limit to the number of regions of interest and comparisons that can be made; the upper limit relates to the size of the region of interest relative the size of the sample. As examples, when a single cell represents a region of interest, then a section may have hundreds to thousands of regions of interest; however, if a tissue section includes only two cell types, then the section may have only two regions of interest (each including only one cell type).

The present disclosure provides a higher degree of multiplexing than is possible with standard immunohistochemical or in situ hybridization methods. Standard immunohistochemical methods allow for maximal simultaneous detection of six to ten protein targets, with three to four protein targets being more typical. Similarly, in situ hybridization methods are limited to simultaneous detection of fewer than ten nucleic acid targets. The present disclosure provides detection of large combinations of nucleic acid targets and/or protein targets from a defined region of a sample. The present disclosure provides an increase in objective measurements by digital quantification and increased reliability and consistency, thereby enabling comparison of results among multiple centers.

Various compositions and methods of the present disclosure are described in full detail herein.

In one aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "two-ended adapter ligation method".

A two ended-adapter ligation method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The probes and samples of the present disclosure are described in further detail herein. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain.

Following contacting the at least on target analyte with the at least one probe, a two-ended adapter ligation method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

Following release of the identifier oligonucleotide, a two-ended adapter ligation method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location, thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

Following collection of the released identifier oligonucleotide, a two-ended adapter ligation method can further comprise: (4) ligating to the released identifier oligonucleotide collected in step (3) at least one nucleic acid adapter.

The nucleic acid adapter can comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated "ROI #1", the nucleic acid adapter would comprise a nucleic acid sequence that corresponds to "ROI #1".

The nucleic acid adapter can also comprise a unique molecular identifier.

The nucleic acid adapter can also comprise a first amplification primer binding site. In other aspects, the nucleic acid adapter can also comprise a second amplification primer binding site.

In some aspects, the nucleic acid adapter can also comprise a constant nucleic acid sequence to minimize ligation bias caused by differences in sequences of particular identifier oligonucleotides.

The nucleic acid adapter can be a partially double-stranded nucleic acid molecule. In aspects in which the nucleic acid adapter is partially double-stranded, the nucleic acid adapter comprises a double-stranded annealed region, a first single-stranded mismatched region, and a second single-stranded mismatched region. The first single-stranded mismatched region and the second single stranded mismatched region can be present on opposing sides of the double-stranded annealed region.

In aspects in which the nucleic acid adapter is partially double-stranded and comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released can be present in the double-stranded annealed region of the nucleic acid adapter.

In aspects in which the nucleic acid adapter is partially double-stranded and comprises a constant nucleic acid sequence to minimize ligation bias, the constant nucleic acid sequence to minimize ligation bias can be present in the double-stranded annealed region of the nucleic acid adapter.

In aspects in which the nucleic acid adapter is partially double-stranded and comprises a unique molecular identifier, the unique molecular identifier can be present in at least one of the first or second single-stranded mismatched regions of the nucleic acid adapter.

In aspects in which the nucleic acid adapter is partially double-stranded and comprises a first and a second amplification primer binding site, the first amplification primer binding site can be present in the first single-stranded mismatched region of the nucleic acid adapter and the second amplification primer binding site can be present in the second single-stranded mismatched region of the nucleic acid adapter.

After ligation of the at least one nucleic acid adapter, a two-ended adapter ligation method can further comprise: (5) amplifying the ligation product produced in step (4) using amplification primers that bind to the first and second amplification primer binding sites; and (6) identifying the released oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the sample.

A two-ended adapter ligation method of the present disclosure can further comprise, prior to step (4), performing an end repair reaction using methods known in the art. The method can also further comprise, prior to step (4), performing a tailing reaction to attach a single nucleotide overhang to the 3' ends of the identifier oligonucleotide using methods known in the art. In aspects, the end repair reaction and the tailing reaction can be performed sequentially or concurrently.

In preferred aspects of a two-ended adapter ligation method, a nucleic acid adapter is ligated to both ends of the released and collected identifier oligonucleotide.

In other aspects of a two-ended adapter ligation method, at least one of the amplification primers used in step (5) to amplify the ligation product produced in step (4) comprises a nucleic acid sequence which identifies the specific location of the tissue sample form which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated "ROI #1", at least one of the amplification primers would comprise a nucleic acid sequence that corresponds to "ROI #1".

FIG. **1** shows a schematic of a preferred aspect of a two-ended adapter ligation method of the present disclosure. In this aspect, the probe comprises a target binding domain comprising an antibody that binds to a target protein. In the upper left panel, the probe binds to the target protein. In the upper right panel, a UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target protein bound to the target binding domain. In the bottom panel, a nucleic acid adapter is ligated to both ends of the identifier oligonucleotide. In this non-limiting example, the nucleic acid adapter is partially double-stranded and comprises a double-stranded annealed region, a first single-stranded mismatched region, and a second single-stranded mismatched region. Present in the double-stranded annealed region is a constant nucleic acid sequence to minimize ligation bias and a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. Present in the first single-stranded mismatched region is a first amplification primer binding site. Present in the second single-stranded mismatched region is a unique molecular identifier and a second amplification primer binding site. Following ligation of the nucleic acid adapters to the identifier oligonucleotide, the product is amplified using amplification primers that bind the first and the second

amplification primer binding sites and sequenced to identify the target protein bound by the probe.

In one aspect, the present disclosure provides a composition of an identifier oligonucleotide dually ligated to two nucleic acid adapters for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide dually ligated to two nucleic acid adapters comprises an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which is capable of identifying a target analyte in a sample. Each end of the identifier oligonucleotide is attached to a nucleic acid adapter molecule, wherein the nucleic acid adapter molecule is partially double-stranded and comprises a double-stranded annealed region, a first single-stranded mismatched region and a second single-stranded mismatched region. The first single-stranded mismatched region and the second single stranded mismatched region are present on opposing sides of the double-stranded annealed region. The double-stranded mismatch region comprises a constant nucleic acid sequence to minimize ligation bias and a nucleic acid sequence nucleic acid sequence which is capable of identifying a specific location of a sample. The first single-stranded mismatched region comprises a first amplification primer binding site. The second single-stranded mismatched region comprises a second amplification primer binding site and a nucleic acid sequence comprising a unique molecular identifier. A schematic of an identifier oligonucleotide dually ligated to two nucleic acid adapters is shown in the bottom panel of FIG. **1**.

In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "one-ended adapter ligation method".

A one-ended adapter ligation method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain. The identifier oligonucleotide can also comprise a first amplification primer binding site. In some aspects, the identifier oligonucleotide also comprises at least one 3' end with a single nucleotide overhang.

Following contacting the at least one target analyte with the at least one probe, a one-ended adapter ligation method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

Following release of the identifier oligonucleotide, a one-ended adapter ligation method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

Following collection of the released identifier oligonucleotide, a one-ended adapter ligation method can further comprise: (4) ligating to the released oligonucleotide collected in step (3) at least one nucleic acid adapter;

The nucleic acid adapter can comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated "ROI #1", the nucleic acid adapter would comprise a nucleic acid sequence that corresponds to "ROI #1". The nucleic acid adapter can also comprise a unique molecular identifier. The nucleic acid adapter can also comprise a second amplification primer binding site.

In some aspects, the nucleic acid adapter can also comprise a constant nucleic acid sequence to minimize ligation bias caused by differences in sequences of particular identifier oligonucleotides. The constant nucleic acid sequence can comprise a cleavable moiety. The cleavable moiety can be enzymatically cleavable. In a non-limiting example, the enzymatically cleavable moiety can be a USER sequence, wherein the USER sequence comprises the sequence GUGUATUG.

The nucleic acid adapter can comprise any combination of the features described above.

The nucleic acid adapter can be a partially double-stranded nucleic acid molecule. In aspects in which the nucleic acid adapter is partially double-stranded, the nucleic acid adapter comprises a double-stranded annealed region and a single-stranded mismatched region.

In aspects in which the nucleic acid adapter is partially double-stranded and comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released can be present in the double-stranded annealed region of the nucleic acid adapter.

In aspects in which the nucleic acid adapter is partially double-stranded and comprises a constant nucleic acid sequence to minimize ligation bias, the constant nucleic acid sequence to minimize ligation bias can be present in the double-stranded annealed region of the nucleic acid adapter.

In aspects in which the nucleic acid adapter is partially double-stranded and comprises a unique molecular identifier, the unique molecular identifier can be present in the single-stranded mismatched region.

In aspects in which the nucleic acid adapter is partially double-stranded and comprises a second amplification primer binding site, the second amplification primer binding site can be present in the single-stranded mismatched region of the nucleic acid adapter.

After ligation of the at least one nucleic acid adapter, a two-ended adapter ligation method can further comprise: (5) amplifying the ligation product produced in step (4); and (6) identifying the released oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the sample.

In other aspects of a one-ended adapter ligation method of the present disclosure, at least one of the amplification primers used in step (5) to amplify the ligation product

produced in step (4) comprises a nucleic acid sequence which identifies the specific location of the tissue sample form which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated "ROI #1", at least one of the amplification primers would comprise a nucleic acid sequence that corresponds to "ROI #1".

FIG. **2** shows a schematic of a preferred aspect of a one-ended adapter ligation method of the present disclosure. In this aspect, the probe comprises a target binding domain that is an antibody that binds to a target protein. In the upper left panel, the probe binds to the target protein. In upper right panel, the UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target protein and a first amplification primer binding site. In this non-limiting example, the identifier oligonucleotide is double-stranded with one strand that comprises three separate nucleic acid molecules. The identifier oligonucleotide also comprises one 3' end with a single nucleotide overhang.

In the bottom panel of FIG. **2**, a nucleic acid adapter is ligated to the end of the identifier oligonucleotide that comprises the 3' single nucleotide overhang. In this non-limiting example, the nucleic acid adapter is partially double-stranded and comprises a double-stranded annealed region and a single-stranded mismatched region. Present in the double-stranded annealed region is a constant nucleic acid sequence to minimize ligation bias and a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. Present in the single-stranded mismatched region is a unique molecular identifier and a second amplification primer binding site. Following ligation of the nucleic acid adapter to the identifier oligonucleotide, the product is amplified using amplification primers that bind to the first and the second amplification primer binding sites and sequenced to identify the target protein bound by the probe.

In one aspect, the present disclosure provides a composition of an identifier oligonucleotide ligated to one nucleic acid adapter for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide ligated to one nucleic acid adapter comprises an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which is capable of identifying a target analyte in a sample and a first amplification primer binding site. One end of the identifier oligonucleotide is attached to a nucleic acid adapter molecule, wherein the nucleic acid adapter molecule is partially double-stranded and comprises a double-stranded annealed region and a single-stranded mismatched region and a second single-stranded mismatched region. The double-stranded mismatch region comprises a constant nucleic acid sequence to minimize ligation bias and a nucleic acid sequence nucleic acid sequence which is capable of identifying a specific location of a sample. The single-stranded mismatched region comprises a second amplification primer binding site. A schematic of an identifier oligonucleotide ligated to one nucleic acid adapter is shown in the bottom panel of FIG. **2**.

In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "templated-primer extension method".

A templated-primer extension method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain. The identifier oligonucleotide can also comprise a first amplification primer binding site.

Following contacting the at least one target analyte with the at least one probe, a templated-primer extension method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

Following release of the identifier oligonucleotide, a templated-primer extension method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

Following collection of the released identifier oligonucleotide, a templated-primer extension method can further comprise: (4) hybridizing to the released identifier oligonucleotide collected in step (3) a single stranded nucleic acid template.

The single stranded nucleic acid template can comprise a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide, thereby allowing for the hybridization of the single stranded nucleic acid template and the collected identifier oligonucleotide.

The single stranded nucleic acid template can also comprise a nucleic acid sequence comprising a unique molecular identifier.

The single stranded nucleic acid template can also comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released.

The single stranded nucleic acid template can also comprise a nucleic acid sequence that is complementary to a second amplification primer binding site.

The single stranded nucleic acid template can comprise any combination of the features described above.

Following hybridization of the identifier oligonucleotide to the single stranded nucleic acid template, a templated-primer extension method can further comprise: (5) extending the identifier oligonucleotide of step (4) to form an extension produce complementary to the single stranded nucleic acid template, wherein the extension product comprises the identifier oligonucleotide and the sequence complementary to the single stranded nucleic acid template; (6) amplifying the extension product of step (6) using amplification primers that hybridize to the first and second

amplification primer binding sites; and (7) identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the sample.

In some aspects, the single stranded nucleic acid template can comprise an affinity molecule. In aspects in which the single stranded nucleic acid template comprises an affinity molecule, a templated-primer extension method can further comprise an affinity purification step between steps (4) and (5).

FIG. **3** shows a schematic of a preferred aspect of a templated-primer extension method of the present disclosure. In this aspect, the probe comprises a target binding domain that is an antibody that binds to a target protein. In the upper left panel, the probe binds to the target protein. In upper right panel, the UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target protein and a first amplification primer binding site. In the lower right panel, the identifier oligonucleotide is hybridized to a single stranded nucleic acid template. In this non-limiting example, the single-stranded nucleic acid template comprises an affinity molecule, a nucleic acid sequence complementary to the unique nucleic acid sequence of the identifier oligonucleotide, a first unique molecular identifier, and a sequence complementary to a second amplification primer biding site. The identifier oligonucleotide is extended to form an extension product complementary to the single stranded nucleic acid template. As shown in the lower left panel, the extension product comprises the identifier oligonucleotide, the nucleic acid sequence complementary to the first unique molecular identifier, and the second amplification primer binding site. Following the extension reaction, the primer extension product is amplified using amplification primers that bind to the first and the second amplification primer binding sites. In this non-limiting example, one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. The amplified product is then sequenced to identify the target protein bound by the probe.

In one aspect, the present disclosure provides a composition of a template-extended identifier oligonucleotide for spatially detecting at least one target analyte in a sample. The template-extended identifier oligonucleotide comprises a first flow cell adapter sequence suitable for sequencing, followed by a first unique molecular identifier, followed by an identifier oligonucleotide, followed by a second unique molecular identifier, followed by a second amplification primer binding site, followed by a third unique molecular identifier, followed by a second flow cell adapter sequence suitable for sequencing. The identifier oligonucleotide comprises a first amplification primer binding site and a unique nucleic acid sequence which is capable of identifying a target analyte in a sample. A schematic of a template-extended identifier oligonucleotide is shown in the bottom panel of FIG. **4**.

In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "short probe hybridization method".

A short probe hybridization method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain.

Following contacting the at least one target analyte with the at least one probe, a short probe hybridization method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

Following release of the identifier oligonucleotide, a short probe hybridization method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

Following collection of the released identifier oligonucleotide, a short probe hybridization method can further comprise: (4) hybridizing to the release identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe.

The first or the second nucleic acid probe can comprise a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. The first or the second nucleic acid probe can also comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. The first or the second nucleic acid probe can also comprise a nucleic acid sequence comprising unique molecular identifier. The first nucleic acid probe can comprise a first amplification primer binding site. The second nucleic acid probe can comprise a second amplification primer binding site.

The first or the second nucleic acid probe can comprise any combination of the features described above. In a preferred aspect depicted in FIG. **5**, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released is located 5' to the first amplification primer binding site and the unique molecular identifier is located 3' to the second amplification primer binding site.

In another preferred aspect depicted in FIG. **6**, the first nucleic acid probe comprises a first amplification primer

binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this same preferred aspect, the second nucleic acid probe comprises a second amplification primer binding site and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and the unique molecular identifier are located 5' to the first amplification primer binding site.

In another preferred aspect depicted in FIG. **7**, the first nucleic acid probe comprises a first amplification primer binding site and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this same preferred aspect, the second nucleic acid probe comprises a second amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and the unique molecular identifier are located 3' to the second amplification primer binding site.

In another preferred aspect depicted in FIG. **15**, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second amplification primer binding site and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence comprising a unique molecular identifier is located 3' to the first amplification binding site.

In another preferred aspect depicted in FIG. **17**, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second amplification primer binding site and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence comprising a unique molecular identifier is located 5' to the first amplification binding site.

The first nucleic acid probe and the second nucleic acid probe can hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping. Alternatively, the first nucleic acid probe and the second nucleic acid probe can hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping.

Following hybridization of the first and the second nucleic acid probe to the identifier oligonucleotide, a short probe hybridization method can further comprise: (5) in the aspect in which the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping, ligating the first and the second nucleic acid probes together, for example, by performing a nick repair reaction. Alternatively, in the aspect in which the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping, the method comprises ligating the first and the second nucleic acid probes together, for example, by performing a gap extension reaction and a nick repair reaction, such that the first and the second nucleic acid probes are ligated together.

Following ligation of the first and the second nucleic acid probe, a short probe hybridization method can further comprise: (6) amplifying the ligation product produced in step (5) using amplification primers that hybridize to the first and second amplification primer binding sites; and (7) identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the sample.

In one aspect, the present disclosure provides a composition of an identifier oligonucleotide-short nucleic acid probe complex for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide-short nucleic acid probe complex comprises an identifier oligonucleotide hybridized to a first nucleic acid probe and a second nucleic acid probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a target analyte in a sample. The first nucleic acid probe comprises a first amplification primer binding site, followed by a unique nucleic acid sequence capable of identifying a specific location in a sample, followed by a region complementary to the identifier oligonucleotide. The second nucleic acid probe comprises a second amplification primer binding site, followed by a nucleic acid sequence comprising a unique molecular identifier, followed by a region complementary to the identifier oligonucleotide. A schematic of an identifier oligonucleotide-short nucleic acid probe complex is depicted in FIG. **5**.

In one aspect, the present disclosure provides a composition of an identifier oligonucleotide-short nucleic acid probe complex for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide-short nucleic acid probe complex comprises an identifier oligonucleotide hybridized to a first nucleic acid probe and a second nucleic acid probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a target analyte in a sample. The first nucleic acid probe comprises a first amplification primer binding site, followed by a nucleic acid sequence comprising a unique molecular identifier, followed by a region complementary to the identifier oligonucleotide, wherein the nucleic acid sequence comprising a unique molecular identifier is located 3' to the first amplification primer binding site. The second nucleic acid probe comprises a second amplification primer binding site followed by a region complementary to the identifier oligonucleotide. A schematic of an identifier oligonucleotide-short nucleic acid probe complex is depicted in FIG. **15**.

In one aspect, the present disclosure provides a composition of an identifier oligonucleotide-short nucleic acid probe complex for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide-short nucleic acid probe complex comprises an identifier oligonucleotide hybridized to a first nucleic acid probe and a second nucleic acid probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a target analyte in a sample. The first nucleic acid probe comprises a first amplification primer binding site, followed by a nucleic acid sequence comprising a unique molecular identifier, followed by a region complementary to the identifier oligonucleotide, wherein the nucleic acid sequence comprising a unique molecular identifier is located

5' to the first amplification primer binding site. The second nucleic acid probe comprises a second amplification primer binding site followed by a region complementary to the identifier oligonucleotide. A schematic of an identifier oligonucleotide-short nucleic acid probe complex is depicted in FIG. **17**.

FIG. **16** shows a schematic overview of an exemplary short probe hybridization method of the present disclosure. First at least one target analyte in a sample is contacted with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target domain.

Following contacting the at least one target analyte with the at least one probe, a force is then provided to a location of the sample sufficient to release the identifier oligonucleotide. The identifier oligonucleotide is collected following release, as shown in the top panel of FIG. **16**.

As shown in the second panel from the top of FIG. **16**, the released identifier oligonucleotide is then hybridized to a first nucleic acid probe and a second nucleic acid probe. In this non-limiting example, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. The nucleic acid sequence comprising the unique molecular identifier is located 3' to the first amplification primer binding site. The second nucleic acid probe comprises a second amplification primer binding site and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this non-limiting example, the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping. Following hybridization to the identifier oligonucleotide, the first and second probe are ligated together, for example, by performing a nick repair reaction.

Following ligation of the first and second nucleic acid probes, the ligation product is amplified via PCR using amplification primers that hybridize to the first and second amplification primer binding sites. As shown in the second panel from the bottom of FIG. **16**, the amplification primer that hybridizes to the second amplification primer binding site comprises a first flow cell binding site, a first nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the second amplification primer binding site. The amplification primer that hybridizes to the first amplification primer binding site comprises a second flow cell binding site, a second nucleic acid sequence which identifies the specific location from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the first amplification primer binding site. The PCR product shown in the bottom panel of FIG. **16** is then sequenced to identify the released oligonucleotide, thereby spatially detecting the at least one target analyte in the sample.

FIG. **18** shows a schematic overview of an exemplary short probe hybridization method of the present disclosure. First, at least one target analyte in a sample is contacted with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target domain.

Following contacting the at least one target analyte with the at least one probe, a force is then provided to a location of the sample sufficient to release the identifier oligonucleotide. The identifier oligonucleotide is collected following release, as shown in the top panel of FIG. **18**.

As shown in the second panel from the top of FIG. **18**, the released identifier oligonucleotide is then hybridized to a first nucleic acid probe and a second nucleic acid probe. In this non-limiting example, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. The nucleic acid sequence comprising the unique molecular identifier is located 5' to the first amplification primer binding site. The second nucleic acid probe comprises a second amplification primer binding site and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this non-limiting example, the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping. Following hybridization to the identifier oligonucleotide, the first and second probe are ligated together, for example, by performing a nick repair reaction.

Following ligation of the first and second nucleic acid probes, the ligation product is amplified via PCR using amplification primers that hybridize to the first and second amplification primer binding sites. As shown in the second panel from the bottom of FIG. **18**, the amplification primer that hybridizes to the second amplification primer binding site comprises a first flow cell binding site, a first nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the second amplification primer binding site. The amplification primer that hybridizes to the first amplification primer binding site comprises a second flow cell binding site, a second nucleic acid sequence which identifies the specific location from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the first amplification primer binding site. The PCR product shown in the bottom panel FIG. **18** is then sequenced to identify the released oligonucleotide, thereby spatially detecting the at least one target analyte in the sample.

In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "long probe hybridization method".

A long probe hybridization method of the present disclosure can comprise: (1) contacting at least one target analyte

in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain.

Following contacting the at least one target analyte with the at least one probe, a long probe hybridization method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

Following release of the identifier oligonucleotide, a long probe hybridization method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

Following collection of the released identifier oligonucleotide(s), a long probe hybridization method can further comprise: (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe.

The first or the second nucleic acid probe can comprise a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. The first or the second nucleic acid probe can also comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released.

The first or the second nucleic acid probe can also comprise a first unique molecular identifier. The first or the second nucleic acid probe can also comprise a second unique molecular identifier. The first or the second nucleic acid probe can also comprise a third unique molecular identifier.

The first nucleic acid probe can comprise a first amplification primer binding site.

The first nucleic acid probe can also comprise a first flow cell binding site. The second nucleic acid probe can comprise a second flow cell binding site.

The first and the second nucleic acid probes can comprise any combination of the features described above. In a preferred aspect depicted in FIG. **8**, the first nucleic acid probe comprises a first flow cell binding site, a first unique molecular identifier, a first amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second flow cell binding site, a second unique molecular identifier, a third unique molecular identifier and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and the first unique molecular identifier are located 5' to the first flow cell binding site and the second and the third unique molecular identifiers are located 3' to the second flow cell binding site.

In another preferred aspect depicted in FIG. **9**, the first nucleic acid probe comprises a first flow cell binding site, a first unique molecular identifier, a second unique molecular identifier, a first amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second flow cell binding site, a third unique molecular identifier and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the first unique molecular identifier, the second unique molecular identifier and the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released are located 5' to the first flow cell binding site and the third unique molecular identifier is located 3' to the second flow cell binding site.

In another preferred aspect depicted in FIG. **10**, the first nucleic acid probe comprises a first flow cell binding site, a first unique molecular identifier, a first amplification primer binding site and a nucleic acid sequence complementary to the identifier oligonucleotide. In this same preferred aspect, the second nucleic acid probe comprises a second flow cell binding site, a second unique molecular identifier, a third unique molecular identifier, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the first unique molecular identifier is located 5' to the first flow cell binding site and the second unique molecular identifier, the third unique molecular identifier, and the nucleic acid sequence which identifies the specific location of the sample form which the identifier oligonucleotide was released are located 3' to the second amplification primer binding site.

The first nucleic acid probe and the second nucleic acid probe can hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping. Alternatively, the first nucleic acid probe and the second nucleic acid probe can hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping.

Following hybridization of the first and the second nucleic acid probe to the identifier oligonucleotide, a long probe hybridization method can further comprise: (5) in the aspect in which the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping, performing a nick repair reaction such that the first and the second nucleic acid probes are ligated together. Alternatively, in the aspect in which the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping, the method comprises performing a gap extension and a nick repair reaction such that the first and the second nucleic acid probes are ligated together.

The name method can further comprise: (6) amplifying the ligation product produced in step (5) using amplification

primers that hybridize to the first and second amplification primer binding sites; and (7) identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the sample.

In one aspect, the present disclosure provides a composition of an identifier oligonucleotide-long nucleic acid probe complex for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide-long nucleic acid probe complex comprises an identifier oligonucleotide hybridized to a first nucleic acid probe and a second nucleic acid probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a target analyte in a sample. The first nucleic acid probe comprises a first flow cell binding site suitable for sequencing, followed by a first unique molecular identifier, followed by a first amplification primer binding site, followed by a unique nucleic acid sequence capable of identifying a specific location in a sample, followed by a region complementary to the identifier oligonucleotide. The second nucleic acid probe comprises a second flow cell binding site, followed by a second unique molecular identifier, followed by a third unique molecular identifier, followed by a region complementary to the identifier oligonucleotide. A schematic of an identifier oligonucleotide-short nucleic acid probe complex is depicted in FIG. **8**.

In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "direct PCR method".

A direct PCR method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain. The identifier oligonucleotide can also comprise a first amplification primer binding site, a second amplification primer binding site, or a unique molecular identifier. The identifier oligonucleotide can comprise any combination of these features. Any of these features can also be flanked by regions comprising constant nucleic acid sequences of about 1 nucleotide to about 10 nucleotides.

Following contacting the at least one analyte with the at least one probe, a direct PCR method further comprises: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

A direct PCR method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

Following release of the identifier oligonucleotide, a direct PCR method can further comprise: (4) amplifying the released identifier oligonucleotide using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site. In some aspects, at least one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated "ROI #1", at least one of the amplification primers would comprise a nucleic acid sequence that corresponds to "ROI #1". In still other aspects, at least one of the amplification primers comprises a unique molecular identifier.

Following amplification, a direct PCR method of the present disclosure can further comprise: (5) identifying the released oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the sample.

FIG. **11** shows a schematic of a preferred aspect of a direct PCR method of the present disclosure. In this aspect, the probe comprises a target binding domain comprising a nucleic acid sequence that is complementary to a target nucleic acid. In the upper panel, the probe hybridizes to the target nucleic acid. In the lower panel, a UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a first amplification primer binding site, a second amplification primer binding site, a unique molecular identifier, and a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain. Located between these four features are constant spacer regions that are 3 nucleotides in length. The identifier oligonucleotide is double-stranded and comprises a strand that comprises 3 separate nucleic acid molecules. After release, the identifier oligonucleotide is amplified using a first amplification primer that hybridizes to the first amplification primer binding site and comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a second amplification primer that hybridizes to the second amplification primer binding site. The amplified product is then sequenced to identify the target nucleic acid bound by the probe.

FIG. **19** shows a schematic of a preferred aspect of a direct PCR method of the present disclosure. In this aspect, the identifier oligonucleotide comprises a first amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier, a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain and a second amplification primer binding site, as shown in the top panel of FIG. **19**. The identifier oligonucleotide is amplified using amplification primers that hybridize to the first and second amplification primer binding sites. As shown in the middle panel of FIG. **19**, the amplification primer that hybridizes to the second amplification primer binding site comprises a first flow cell binding site, a first nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence comple-

mentary to the second amplification primer binding site. The amplification primer that hybridizes to the first amplification primer binding site comprises a second flow cell binding site, a second nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the first amplification primer binding site. The PCR product shown in the bottom panel of FIG. **19** is sequenced to identify the released oligonucleotide, thereby spatially detecting the at least one target analyte in the sample.

In one aspect, the present disclosure provides a composition of a direct-PCR compatible identifier oligonucleotide for spatially detecting at least one target analyte in a sample. A direct-PCR compatible identifier oligonucleotide comprises a first amplification primer binding site, followed a unique nucleic acid sequence which is capable of identifying a target analyte in a sample, followed by a unique molecular identifier, followed by a second amplification primer binding site. A schematic of a direct-PCR compatible identifier oligonucleotide is depicted in the lower panel of FIG. **11**.

In one aspect, the present disclosure provides a composition of a direct-PCR compatible identifier oligonucleotide for spatially detecting at least one target analyte in a sample. A direct-PCR compatible identifier oligonucleotide comprises a first amplification primer binding site, followed a nucleic acid sequence comprising a unique molecular identifier, followed by a unique nucleic acid sequence which is capable of identifying a target analyte in a sample, followed by a second amplification primer binding site. A schematic of a direct-PCR compatible identifier oligonucleotide is shown in the top panel of FIG. **19**.

In some aspects of the methods of the present disclosure at least one, or at least two, or at least three, or at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least ten, or at least eleven, or at least twelve, or at least thirteen, or at least fourteen, or at least fifteen, or at least sixteen, or at least seventeen, or at least eighteen, or at least nineteen, or at least twenty, or at least thirty, or at least forty, or at least fifty, or at least sixty, or at least seventy, or at least eighty, or at least ninety, or at least one hundred probes can been to a single target analyte. As used herein, the term "tiling" is used to describe when more than one probe of the present disclosure is bound to a target analyte. The top panel of FIG. **27** shows the tiling of probes onto a target RNA. Tiling multiple probes onto a target analyte means that each target analyte will be individually detected multiple times, increasing the overall accuracy of the measurement. In a non-limiting example, as shown in the bottom panel of FIG. **27**, in the case where 10 probes are tiled onto a single target RNA, one of the probes may be incorrectly detected too many times (outlier high count probe), while another probe may be incorrectly detected too few times (outlier low count probe). However, the other 8 probes may be detected at a similar level, indicating that the two outliers should be discarded during analysis and the signals from the 8 probes used to generate a more accurate measurement of the abundance of the target RNA.

The present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as an "enzyme free method".

An enzyme free method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. An identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain. An identifier oligonucleotide can also comprise a capture probe binding site.

Following contacting at least one target analyte with at least one probe, an enzyme free method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

Following release of an identifier oligonucleotide, an enzyme free method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

Following collection of a released identifier oligonucleotide, an enzyme free method can further comprise: (4) hybridizing to a released identifier oligonucleotide a capture probe.

A capture probe can comprise a region complementary to the capture probe binding site. A capture probe can also comprise an affinity molecule.

Following hybridization of a capture probe, an enzyme free method can further comprise: (5) Identifying a released identifier oligonucleotide by sequencing the hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

A hybridized product produced in step (4) can be sequenced using an enzyme free method of sequencing. Enzyme-free methods of sequencing have been described in, e.g., US2014946386 and U.S. Ser. No. 15/819,151 (U.S. Pat. No. 10,415,080), each of which is incorporated herein by reference in its entirety.

FIG. **12** shows a schematic of a preferred aspect of an enzyme free method of the present disclosure. In this aspect, the probe comprises a target binding domain comprising a nucleic acid sequence that is complementary to a target nucleic acid. In the top panel, the probe hybridizes to the target nucleic acid. In the middle panel, a UV photocleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target nucleic acid bound to the target binding domain and a capture probe binding site. After release, the identifier oligonucleotide is hybridized to a capture probe, as depicted in the bottom panel. The capture probe comprises a nucleic acid sequence complementary to the capture probe binding

site and an affinity molecule. The hybridized product is then sequenced using enzyme free sequencing methods to identify the target nucleic acid bound by the probe.

In one aspect, the present disclosure provides a composition of a hybridized identifier oligonucleotide-capture probe complex for spatially detecting at least one target analyte in a sample. A hybridized identifier oligonucleotide-capture probe complex comprises an identifier oligonucleotide hybridized to a capture probe. An identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a specific a target analyte in a sample and a capture probe binding site. A capture probe comprises an affinity molecule and a region complementary to the capture probe binding site. A schematic of a hybridized identifier oligonucleotide-capture probe complex is depicted in the bottom of panel FIG. **12**.

The present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "multiplexed enzyme free method".

A multiplexed enzyme free method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain. The identifier oligonucleotide can also comprise a capture probe binding site. The identifier oligonucleotide can also comprise a multiplexing probe binding site.

Following contacting the at least one target analyte with the at least one probe, a multiplexed enzyme free method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

Following release of the identifier oligonucleotide, a multiplexed enzyme free method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

Following collection of the released identifier oligonucleotide, a multiplexed enzyme free method can further comprise: (4) hybridizing to the released identifier oligonucleotide a capture probe and a multiplexing probe.

A capture probe can comprise a region complementary to the capture probe binding site. A capture probe can also comprise an affinity molecule.

A multiplexing probe can comprise a region complementary to the multiplexing probe binding site. A multiplexing probe can also comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a region complementary to the multiplexing probe binding site.

Following hybridization of a capture probe and a multiplexing probe, a multiplexed enzyme free method can further comprise: (5) Identifying the released identifier oligonucleotide by sequencing the hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

A hybridized product produced in step (4) can be sequenced using an enzyme free method of sequencing. Enzyme-free methods of sequencing have been described in, e.g., US2014946386 and U.S. Ser. No. 15/819,151, each of which is incorporated herein by reference in its entirety.

FIG. **13** shows a schematic of a preferred aspect of a multiplexed enzyme free method of the present disclosure. In this aspect, the probe comprises a target binding domain comprising a nucleic acid sequence that is complementary to a target nucleic acid. In the top panel, the probe hybridizes to the target nucleic acid. In the middle panel, a UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain, a capture probe binding site, and a multiplexing probe binding site, as shown in the middle panel. After release, the identifier oligonucleotide is hybridized to a capture probe and a multiplexing probe as shown in the lower panel. The capture probe comprises a nucleic acid sequence complementary to the capture probe binding site and an affinity molecule. The multiplexing probe comprises a nucleic acid sequence complementary to the multiplexing probe binding site and a nucleic acid sequence which identifies the specific location of a sample from which the identifier oligonucleotide was released. The hybridized product is then sequenced using enzyme free sequencing methods to identify the target nucleic acid bound by the probe.

In one aspect, the present disclosure provides a composition of a hybridized identifier oligonucleotide-capture probe-multiplex probe complex for spatially detecting at least one target analyte in a sample. A hybridized identifier oligonucleotide-capture probe-multiplex probe complex comprises an identifier oligonucleotide hybridized to a capture probe and a multiplexing probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a specific a target analyte in a sample, a capture probe binding site and a multiplexing probe binding site. The capture probe comprises an affinity molecule and a region complementary to the capture probe binding site. The multiplexing probe comprises a nucleic acid sequence which identifies the specific location of a sample from which the identifier oligonucleotide was released and a region complementary to the multiplexing probe binding site. A schematic of a hybridized identifier oligonucleotide-capture probe-multiplex probe complex is depicted in the bottom panel of FIG. **13**.

FIG. **20** is an exemplary schematic of overview of the methods of the present disclosure. First, a sample on a microscope slide is contacted with a plurality of probes of the present disclosure (step **1** in FIG. **20**). The slide is then imaged and particular regions of interest (ROIs) are selected (step **2** in FIG. **20**). A specific ROI is then illuminated by UV

light to release identifier oligonucleotides from probes bound within the ROI. The released identifier oligonucleotides are then collected via aspiration with a microcapillary. Following aspiration, the identifier oligonucleotides are transferred to a particular well within a 96 well plate. Steps 4 and 5 are then repeated for each ROI identified in step **2**. After all ROIs have been illuminated and all released identifier oligonucleotides collected, the identifier oligonucleotides are sequenced using next generation sequencing methods to spatially detect at least one target analyte in the sample.

As described in the preceding, the present disclosure provides probes for the compositions and methods of spatially detecting at least one target analyte in a sample. The present disclosure provides probes comprising a target binding domain and an identifier oligonucleotide. The target binding domain is a region of the probe that specifically binds to at least one target analyte in a sample.

Probes of the present disclosure can be used for spatially detecting a target nucleic acid. In this aspect, the target binding domain can be a target nucleic acid-binding region. The target nucleic acid-binding region is preferably at least 15 nucleotides in length, and more preferably is at least 20 nucleotides in length. In specific aspects, the target nucleic acid-binding region is approximately 10 to 500, 20 to 400, 25, 30 to 300, 35, 40 to 200, or 50 to 100 nucleotides in length. Probes and methods for binding and identifying a target nucleic acid have been described in, e.g., US2003/0013091, US2007/0166708, US2010/0015607, US2010/0261026, US2010/0262374, US2010/0112710, US2010/0047924, and US2014/0371088, each of which is incorporated herein by reference in its entirety.

The target nucleic acid-binding region can directly hybridize to a target nucleic acid present in a sample. Alternatively, the probes of the present disclosure can indirectly hybridize to a target nucleic acid present in a sample (via an intermediary oligonucleotide). FIG. **14** illustrates a probe (or composition) of this aspect. The probe includes a target nucleic-acid binding domain which binds to a synthetic oligonucleotide (the intermediary oligonucleotide) that in turn binds to a target nucleic acid in a biological sample. It could be said that the intermediary oligonucleotide is a probe, as defined herein, since it comprises a nucleic acid backbone and is capable of binding a target nucleic acid. In these aspects, a probe's target nucleic acid-binding region hybridizes to a region of an intermediary oligonucleotide (i.e., a synthetic oligonucleotide) which is different from the target nucleic acid present in a sample. Thus, the probe's target binding region is independent of the ultimate target nucleic acid in the sample. This allows economical and rapid flexibility in an assay design, as the target (present in a sample)-specific components of the assay are included in inexpensive and widely-available synthetic DNA oligonucleotides rather than the more expensive probes. Such synthetic oligonucleotides are simply designed by including a region that hybridizes to the target nucleic acid present in a sample and a region that hybridizes to a probe. Therefore, a single set of indirectly-binding probes can be used to detect an infinite variety of target nucleic acids (present in a sample) in different experiments simply by replacing the target-specific (synthetic) oligonucleotide portion of the assay.

A target nucleic acid may be DNA or RNA and preferably messenger RNA (mRNA) or miRNA.

Probes of the present disclosure can be used for detecting a target protein. In this aspect, the target binding domain can be a target protein-binding region. A target protein-binding region includes molecules or assembles that are designed to bind to at least one target protein, at least one target protein surrogate, or both and can, under appropriate conditions, form a molecular complex comprising the probe and the target protein. The target-protein binding region can include an antibody, a peptide, an aptamer, or a peptoid. The antibody can be obtained from a variety of sources, including but not limited to polyclonal antibody, monoclonal antibody, monospecific antibody, recombinantly expressed antibody, humanized antibody, plantibodies, and the like. The terms protein, polypeptide, peptide, and amino acid sequence are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids or synthetic amino acids. The terms also encompass an amino acid polymer that has been modified, for example, by disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein the term amino acid refers to either natural and/or unnatural or synthetic amino acids, including but not limited to glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. Probes and methods for binding and identifying a target protein have been described, e.g., in US2011/0086774, the contents of which is incorporated herein by reference in its entirety.

An identifier oligonucleotide is a nucleic acid molecule that identifies the target analyte bound to the target binding domain. The identifier oligonucleotide comprises a unique nucleic acid sequence that identifies the target analyte bound to the target binding domain of the probe. In a non-limiting example, a probe with a target binding domain that binds to the protein P53 comprises an identifier oligonucleotide with a unique nucleic acid sequence that corresponds to P53, while a probe with a target binding domain that binds to the protein P97 comprises an identifier oligonucleotide with a unique nucleic acid sequence that corresponds to P97.

An identifier oligonucleotide can be DNA, RNA, or a combination of DNA and RNA.

In some aspects, an identifier oligonucleotide can comprise at least one amplification primer binding site. An amplification primer binding site is a nucleic acid sequence capable of binding to an amplification primer. An amplification primer can be used to amplify the nucleic molecule to which it is bound using methods known in the art, including, but not limited to, polymerase chain reaction (PCR).

In some aspects, an identifier oligonucleotide can comprise at least one unique molecular identifier.

An identifier oligonucleotide can be a single-stranded, a double-stranded, or a partially double-stranded nucleic acid molecule. In the aspects in which an identifier oligonucleotide is double-stranded or partially double-stranded, at least one of the two strands can comprise at least two separate nucleic acid molecules which, without being bound by theory, allows for denaturing of the identifier oligonucleotide at lower temperatures.

An identifier oligonucleotide can also comprise at least one 3' end that comprises a single nucleotide overhang.

An identifier oligonucleotide can also comprise a capture probe binding site. A capture probe binding site is a nucleic acid sequence to which a capture probe can bind.

A capture probe of the present disclosure can comprise a nucleic acid sequence complementary to a capture probe binding site. A capture probe can also comprise an affinity molecule.

An identifier oligonucleotide can also comprise a multiplexing probe binding site. A multiplexing probe binding site is a nucleic acid sequence to which a multiplexing probe can bind.

A multiplexing probe of the present disclosure can comprise a nucleic acid sequence complementary to a multiplexing probe binding site. A multiplexing probe can also comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which an identifier oligonucleotide was released.

A probe of the present disclosure can include a region which permits the release of an identifier oligonucleotide following the application of a suitable force. In one non-limited example, the region is a cleavable motif (e.g., a restriction enzyme site or cleavable linker). The cleavable motif allows release of an identifier oligonucleotide from a bound target nucleic acid or protein and the identifier oligonucleotide can then be collected and detected. The region which permits the release of an identifier oligonucleotide can be positioned between the target-binding domain and the identifier oligonucleotide, allowing for the release of the identifier oligonucleotide from the target binding domain. An identifier oligonucleotide is said to be releasable when it can be separated (i.e., cleaved and released) from the remainder of the probe. Examples of cleavable motives include but are not limited to photo-cleavable linkers. Photo-cleavable linkers can be cleaved by light provided by a suitable coherent light source (e.g., a laser and a UV light source) or a suitable incoherent light source (e.g., an arc-lamp and a light-emitting diode (LED)).

In some aspects, the identifier oligonucleotide is collected from a solution proximal to, e.g., at least immediately above or surrounding, the point at which the identifier oligonucleotide is released or the at least one cell. The proximal solution may be collected by aspirating, e.g., via a pipette, a capillary tube, a microarray pin, a flow cell comprising holes, or another suitable aspirating system known in the art or any combination thereof. The capillary tube may comprise an optical device capable of transmitting a light force, e.g., UV light, to the at least one cell. The pipette or a microarray pin may be attached to an array comprising a plurality of pipettes or microarray pins. The proximal solution may comprise an anionic polymer, e.g., dextran sulfate, and/or salmon sperm DNA and/or the collected signal oligonucleotide may be added to a solution comprising an anionic polymer, e.g., dextran sulfate, and/or salmon sperm DNA. Other non-specific blocking agents known in the art in addition to or instead of salmon sperm DNA may be used.

In some aspects, the identifier oligonucleotide is collected from a tissue, at least one cell or proximal to the point at which the identifier oligonucleotide is released via liquid laminar, turbulent, or transitional flow. The flow may be via a channel, e.g., having 25 to 500 m depth between the tissue and a fluidic device or impermeable barrier placed over the tissue.

In aspects where the target-binding domain of a probe is an antibody, the probe can be prepared using a cysteine bioconjugation method that is stable, site-specific to, preferably, the antibody's hinge-region heavy-chain. This preparation method provides relatively controllable identifier oligonucleotides to antibody stoichiometric ratios. A probe can comprise a plurality (i.e., more than one, e.g., 2, 3, 4, 5, or more) identifier oligonucleotides per antibody. Generally, "heavier" probes, which comprise 3 or 4 identifier oligonucleotides per antibody, are significantly less sensitive than antibodies lacking an identifier oligonucleotide or "lighter" probes, which comprise 1 or 2 identifier oligonucleotides per antibody.

In aspects, probes are provided to a sample at concentrations typically less than that used for immunohistochemistry (IHC) or for in situ hybridization (ISH). Alternately, the concentration may be significantly less than that used for IHC or ISH. For example, the probe concentration may be 2 fold less, 5 fold less, 10 fold less, 20 fold less, 25 fold less, 30 fold less, 50 fold less, 60 fold less, 70 fold less, 80 fold less, 90 fold less, 100 fold less, 200 fold less, 300 fold less, 400 fold less, 500 fold less, 600 fold less, 700 fold less, 800 fold less, 900 fold less, 1000 fold less, 2000 fold less, or less and any number in between. In aspects, probes are provided at a concentration of 100 nM, 70 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM, 9 nM, 8 nM, 7 nM, 6 nM, 5 nM, 4 nM, 3 nM, 2 nM, 1 nM, 0.9 nM, 0.8 nM, 0.7 nM, 0.6 nM, 0.5 nM, 0.4 nM, 0.3 nM, 0.2 nM, 0.1 nM, 0.09 nM, 0.08 nM, 0.07 nM, 0.06 nM, 0.05 nM, 0.04 nM, 0.03 nM, 0.02 nM, 0.01 nM, and less and any concentration in between.

Background noise, during protein detection, can be reduced by performing a negative purification of the intact probe molecule. This can be done by conducting an affinity purification of the antibody or photo-cleavable linker after collection of eluate from a region of interest. Normally, released signal oligonucleotides will not be pulled out of solution. A protein-G or -O mechanism in a pipet tip, tube, or plate can be employed for this step. Such devices and reagents commercially available.

Background noise, during nucleic acid detection, can be reduced by performing a negative purification of the intact probe molecule. This can be done by conducting an affinity purification of the target binding domain or photo-cleavable linker after collection of eluate from a region of interest. Normally, released signal oligonucleotides will not be pulled out of solution. To assist in the negative purification, a universal purification sequence may be included in a probe, e.g., in the target binding domain.

Protein-targeting probes and nucleic acid-targeting probes may be applied simultaneously as long as conditions allow for binding of both a protein target and a nucleic acid target. Alternately, protein-targeting probes and nucleic acid-targeting probes may be applied sequentially when conditions allowing for binding of both a protein target and a nucleic acid target are not possible.

A set of probes is synonymous with a composition of probes. A set of probes includes at least one species of probes, i.e., directed to one target. A set of probes preferably includes at least two, e.g., 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 or more species of probes. A probe set may include one or multiple copies of each species of probe.

A first set of probes only may be applied to a sample. Alternately, a second set (or higher number) of probes may be later applied to the sample. The first set and second (or higher number) may target only nucleic acids, only proteins, or a combination thereof.

In the present disclosure, two or more targets (i.e., proteins, nucleic acids, or a combination thereof) are detected; 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 or more targets, and any number there between, are detected.

A set of probes may be pre-defined based upon the cell type or tissue type to be targeted. For example, if the tissue is a breast cancer, then the set of probes will include probes

directed to proteins relevant to breast cancer cells (e.g., Her2, EGFR, and PR) and/or probes directed to proteins relevant to normal breast tissues. Additionally, the set of probes may be pre-defined based upon developmental status of a cell or tissue to be targeted. Alternately, the set of probes may be pre-defined based upon subcellular localizations of interest, e.g., nucleus, cytoplasm, and membrane. For example, antibodies directed to Foxp3, Histone H3, or P-S6 label the nucleus, antibodies directed to CD3, CD4, PD-1, or CD45RO label the cytoplasm, and antibodies directed to PD-L1 label membranes.

A probe may be chemically synthesized or may be produced biologically using a vector into which a nucleic acid encoding the probe has been cloned.

Any probe or set of probes described herein may be used in methods and kits of the present disclosure.

For the herein-described probes, association of a unique nucleic acid sequence to a specific target nucleic acid or target protein is not fixed.

As described in the preceding, probes of the present disclosure can be used to detect a target nucleic acid or target protein present in any sample, e.g., a biological sample. As will be appreciated by those in the art, the sample may comprise any number of things, including, but not limited to: cells (including both primary cells and cultured cell lines) and tissues (including cultured or explanted). In aspects, a tissue sample (fixed or unfixed) is embedded, serially sectioned, and immobilized onto a microscope slide. As is well known, a pair of serial sections will include at least one cell that is present in both serial sections. Structures and cell types, located on a first serial section will have a similar location on an adjacent serial section. The sample can be cultured cells or dissociated cells (fixed or unfixed) that have been immobilized onto a slide. A sample can be a formalin-fixed paraffin-embedded (FFPE) tissue sample.

In aspects, a tissue sample is a biopsied tumor or a portion thereof, i.e., a clinically-relevant tissue sample. For example, the tumor may be from a breast cancer. The sample may be an excised lymph node.

The sample can be obtained from virtually any organism including multicellular organisms, e.g., of the plant, fungus, and animal kingdoms; preferably, the sample is obtained from an animal, e.g., a mammal. Human samples are particularly preferred.

In some aspects, the probes, compositions, methods, and kits described herein are used in the diagnosis of a condition. As used herein the term diagnose or diagnosis of a condition includes predicting or diagnosing the condition, determining predisposition to the condition, monitoring treatment of the condition, diagnosing a therapeutic response of the disease, and prognosis of the condition, condition progression, and response to particular treatment of the condition. For example, a tissue sample can be assayed according to any of the probes, methods, or kits described herein to determine the presence and/or quantity of markers of a disease or malignant cell type in the sample (relative to the non-diseased condition), thereby diagnosing or staging a disease or a cancer.

In general, samples attached to a slide can be first imaged using fluorescence (e.g., fluorescent antibodies or fluorescent stains (e.g., DAPI)) to identify morphology, regions of interest, cell types of interest, and single cells and then expression of proteins and/or nucleic acids can be digitally counted from the sample on the same slide.

Compositions and kits of the present disclosure can include probes and other reagents, for example, buffers and other reagents known in the art to facilitate binding of a protein and/or a nucleic acid in a sample, i.e., for performing hybridization reactions.

A kit also will include instructions for using the components of the kit, including, but not limited to, information necessary to hybridize labeled oligonucleotides to a probe, to hybridize a probe to a target-specific oligonucleotide, to hybridize a target-specific oligonucleotide to a target nucleic acid and/or to hybridize a probe to target protein.

A region of interest may be a tissue type present in a sample, a cell type, a cell, or a subcellular structure within a cell.

Together, a comparison of the identity and abundance of the target proteins and/or target nucleic acids present in a first region of interest (e.g., tissue type, a cell type (including normal and abnormal cells), and a subcellular structure within a cell) and the identity and abundance of the target proteins and/or target nucleic acids present in second region of interest or more regions of interest can be made using the methods of the present disclosure.

As described in the preceding, the products produced by the methods of the present disclosure can be used for nucleic acid amplification. In a preferred aspect, the nucleic acid amplification can be solid-phase nucleic acid amplification. Thus, in further aspects the invention provides a method of solid-phase nucleic acid amplification of template polynucleotide molecules which comprises: preparing a library of template polynucleotide molecules which have common sequences at their 5' and 3' ends using the methods of the present disclosure and carrying out a solid-phase nucleic acid amplification reaction wherein said template polynucleotide molecules are amplified. Compositions and methods for nucleic acid amplification and sequencing have been described in, e.g., U.S. Pat. No. 9,376,678, which is incorporated herein by reference in its entirety.

The term "solid-phase amplification" as used herein refers to any nucleic acid amplification reaction carried out on or in association with a solid support such that all or a portion of the amplified products are immobilized on the solid support as they are formed. In particular, the term encompasses solid-phase polymerase chain reaction (solid-phase PCR), which is a reaction analogous to standard solution phase PCR, except that one or both of the forward and reverse amplification primers is/are immobilized on the solid support.

Although the invention encompasses "solid-phase" amplification methods in which only one amplification primer is immobilized (the other primer usually being present in free solution), it is preferred for the solid support to be provided with both the forward and the reverse primers immobilized. In practice, there will be a "plurality" of identical forward primers and/or a "plurality" of identical reverse primers immobilized on the solid support, since the PCR process requires an excess of primers to sustain amplification. References herein to forward and reverse primers are to be interpreted accordingly as encompassing a "plurality" of such primers unless the context indicates otherwise.

As will be appreciated by the skilled reader, any given PCR reaction requires at least one type of forward primer and at least one type of reverse primer specific for the template to be amplified. However, in certain aspects the forward and reverse primers may comprise template-specific portions of identical sequence, and may have entirely identical nucleotide sequence and structure (including any non-nucleotide modifications). In other words, it is possible to carry out solid-phase amplification using only one type of

primer, and such single-primer methods are encompassed within the scope of the invention. Other aspects may use forward and reverse primers which contain identical template-specific sequences but which differ in some other structural features. For example one type of primer may contain a non-nucleotide modification which is not present in the other.

In other aspects of the invention the forward and reverse primers may contain template-specific portions of different sequence.

Amplification primers for solid-phase PCR are preferably immobilized by covalent attachment to the solid support at or near the 5' end of the primer, leaving the template-specific portion of the primer free for annealing to its cognate template and the 3' hydroxyl group free for primer extension. Any suitable covalent attachment means known in the art may be used for this purpose. The chosen attachment chemistry will depend on the nature of the solid support, and any derivatization or functionalization applied to it. The primer itself may include a moiety, which may be a non-nucleotide chemical modification, to facilitate attachment. In one particularly preferred aspect the primer may include a sulphur-containing nucleophile, such as phosphorothioate or thiophosphate, at the 5' end. In the case of solid-supported polyacrylamide hydrogels (as described below), this nucleophile will bind to a "C" group present in the hydrogel. The most preferred means of attaching primers and templates to a solid support is via 5' phosphorothioate attachment to a hydrogel comprised of polymerised acrylamide and N-(5-bromoacetamidylpentyl)acrylamide (BRAPA).

The terms "cluster" and "colony" are used interchangeably herein to refer to a discrete site on a solid support comprised of a plurality of identical immobilized nucleic acid strands and a plurality of identical immobilized complementary nucleic acid strands. The term "clustered array" refers to an array formed from such clusters or colonies. In this context the term "array" is not to be understood as requiring an ordered arrangement of clusters.

The invention also encompasses methods of sequencing the amplified nucleic acids generated by solid-phase amplification. Thus, the invention provides a method of nucleic acid sequencing comprising amplifying a library of nucleic acid templates by the methods of the present disclosure described above, using solid-phase amplification as described above to amplify this library on a solid support, and carrying out a nucleic acid sequencing reaction to determine the sequence of the whole or a part of at least one amplified nucleic acid strand produced in the solid-phase amplification reaction.

Sequencing, as referred to herein, can be carried out using any suitable "sequencing-by-synthesis" technique, wherein nucleotides are added successively to a free 3' hydroxyl group, resulting in synthesis of a polynucleotide chain in the 5' to 3' direction. The nature of the nucleotide added is preferably determined after each nucleotide addition.

The initiation point for the sequencing reaction may be provided by annealing of a sequencing primer to a product of the whole genome or solid-phase amplification reaction. In this connection, one or both of the adapters added during formation of the template library may include a nucleotide sequence which permits annealing of a sequencing primer to amplified products derived by whole genome or solid-phase amplification of the template library.

The products of solid-phase amplification reactions wherein both forward and reverse amplification primers are covalently immobilized on the solid surface are so-called "bridged" structures formed by annealing of pairs of immobilized polynucleotide strands and immobilized complementary strands, both strands being attached to the solid support at the 5' end. Arrays comprised of such bridged structures provide inefficient templates for nucleic acid sequencing, since hybridization of a conventional sequencing primer to one of the immobilized strands is not favored compared to annealing of this strand to its immobilized complementary strand under standard conditions for hybridization.

In order to provide more suitable templates for nucleic acid sequencing it is preferred to remove substantially all or at least a portion of one of the immobilized strands in the "bridged" structure in order to generate a template which is at least partially single-stranded. The portion of the template which is single-stranded will thus be available for hybridization to a sequencing primer. The process of removing all or a portion of one immobilized strand in a "bridged" double-stranded nucleic acid structure may be referred to herein as "linearization".

Bridged template structures may be linearized by cleavage of one or both strands with a restriction endonuclease or by cleavage of one strand with a nicking endonuclease. Other methods of cleavage can be used as an alternative to restriction enzymes or nicking enzymes, including inter alia chemical cleavage (e.g. cleavage of a diol linkage with periodate), cleavage of abasic sites by cleavage with endonuclease, or by exposure to heat or alkali, cleavage of ribonucleotides incorporated into amplification products otherwise comprised of deoxyribonucleotides, photochemical cleavage or cleavage of a peptide linker.

It will be appreciated that a linearization step may not be essential if the solid-phase amplification reaction is performed with only one primer covalently immobilized and the other in free solution.

In order to generate a linearized template suitable for sequencing it is necessary to remove "unequal" amounts of the complementary strands in the bridged structure formed by amplification so as to leave behind a linearized template for sequencing which is fully or partially single stranded. Most preferably one strand of the bridged structure is substantially or completely removed.

Following the cleavage step, regardless of the method used for cleavage, the product of the cleavage reaction may be subjected to denaturing conditions in order to remove the portion(s) of the cleaved strand(s) that are not attached to the solid support. Suitable denaturing conditions will be apparent to the skilled reader with reference to standard molecular biology protocols.

Denaturation (and subsequent re-annealing of the cleaved strands) results in the production of a sequencing template which is partially or substantially single-stranded. A sequencing reaction may then be initiated by hybridization of a sequencing primer to the single-stranded portion of the template.

Thus, the nucleic acid sequencing reaction may comprise hybridizing a sequencing primer to a single-stranded region of a linearized amplification product, sequentially incorporating one or more nucleotides into a polynucleotide strand complementary to the region of amplified template strand to be sequenced, identifying the base present in one or more of the incorporated nucleotide(s) and thereby determining the sequence of a region of the template strand.

One preferred sequencing method which can be used in accordance with the invention relies on the use of modified nucleotides that can act as chain terminators. Once the modified nucleotide has been incorporated into the growing polynucleotide chain complementary to the region of the template being sequenced there is no free 3'-OH group

available to direct further sequence extension and therefore the polymerase cannot add further nucleotides. Once the nature of the base incorporated into the growing chain has been determined, the 3' block may be removed to allow addition of the next successive nucleotide. By ordering the products derived using these modified nucleotides it is possible to deduce the DNA sequence of the DNA template. Such reactions can be done in a single experiment if each of the modified nucleotides has attached a different label, known to correspond to the particular base, to facilitate discrimination between the bases added at each incorporation step. Alternatively, a separate reaction may be carried out containing each of the modified nucleotides separately.

The modified nucleotides may carry a label to facilitate their detection. Preferably this is a fluorescent label. Each nucleotide type may carry a different fluorescent label. However the detectable label need not be a fluorescent label. Any label can be used which allows the detection of an incorporated nucleotide.

One method for detecting fluorescently labelled nucleotides comprises using laser light of a wavelength specific for the labelled nucleotides, or the use of other suitable sources of illumination. The fluorescence from the label on the nucleotide may be detected by a CCD camera or other suitable detection means.

The invention is not intended to be limited to use of the sequencing method outlined above, as essentially any sequencing methodology which relies on successive incorporation of nucleotides into a polynucleotide chain can be used. Suitable alternative techniques include, for example, Pyrosequencing, FISSEQ (fluorescent in situ sequencing), MPSS (massively parallel signature sequencing) and sequencing by ligation-based methods.

In methods of the present disclosure, the unique nucleic acid sequence present in the identifier oligonucleotide of a probe which identifies the target analyte bound to the target binding domain of the probe can comprise between about 5 nucleotides and about 50 nucleotides. Preferably, the sequence comprises between about 20 nucleotides and about 40 nucleotides. Even more preferably, the sequence comprises about 35 nucleotides. In some preferred aspects, the sequence comprises 10 nucleotides.

In methods of the present disclosure, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released comprises between about 6 nucleotides and about 15 nucleotides. Preferably, the sequence comprises about 12 nucleotides.

In methods of the present disclosure, an amplification primer binding sites comprises between about 18 nucleotides and about 40 nucleotides. Preferably, an amplification primer binding sites comprises about 32 nucleotides.

In some aspects of the methods of the present disclosure, an amplification primer binding site can comprise an i7 sequence, wherein the i7 sequence comprises the sequence set forth in SEQ ID NO: 1.

In some aspects of the methods of the present disclosure, an amplification primer binding site can comprise an i5 sequence, wherein the i5 sequence comprises the sequence set forth in SEQ ID NO: 2.

In some aspects of the methods of the present disclosure, an amplification primer can comprise a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing. Preferably, at least one amplification primer used in the methods of the present disclosure comprises a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3. Preferably still, at least one amplification primer used in the methods of the present disclosure comprises a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

In methods of the present disclosure, a unique molecular identifier can comprise between about 6 nucleotides and about 30 nucleotides. Preferably, a unique molecular identifier can comprise about 15 nucleotides. The terms unique molecular identifier and random molecular tags are used interchangeably herein. Using methods known in that art, unique molecular identifiers are random sequences that can be used to correct for biases in amplification prior to sequencing.

In methods of the present disclosure, a constant nucleic acid sequence to minimize ligation bias comprises between about 1 nucleotide and about 15 nucleotides. Preferably, the constant sequence comprises about 8 nucleotides.

In some aspects, a flow cell binding site can comprise between about 15 to about 40 nucleotides. A flow cell binding site can comprise about 29 nucleotides. A flow cell binding site can comprise about 24 nucleotides.

In some aspects, a target binding domain can comprise between about 10 to about 70 nucleotides. A target binding domain can comprise between about 30 to about 55 nucleotides. A target binding domain can comprise between about 35 to about 50 nucleotides.

In some aspects, a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain can comprise between about 20 to about 40. A unique nucleic acid sequence which identifies the target analyte bound to the target binding domain can comprise about 25 nucleotides, or about 35 nucleotides, or about 12 nucleotides.

In some aspects, an amplification primer binding site can comprise between about 20 to about 50 nucleotides. An amplification primer binding site can comprise about 33 nucleotides, or about 34 nucleotides.

In some aspects, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can comprise between about 1 to about 20 nucleotides. A nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can comprise about 8 nucleotides.

In some aspects, a nucleic acid sequence comprising a unique molecular identifier can comprise between about 5 to about 20 nucleotides. A nucleic acid sequence comprising a unique molecular identifier can comprise about 14 nucleotides.

As used herein, the terms “region of interest” and “ROI” are used in their broadest sense to refer to a specific location within a sample that is to be analyzed using the methods of the present disclosure.

As used herein, the term “adjacent” can mean within about 1 nucleotide, or within about 2 nucleotides, or within about 3 nucleotides, or within about 4 nucleotides, or within about 5 nucleotides, or within about 6 nucleotides, or within about 7 nucleotides, or within about 8 nucleotides, or within about 9 nucleotides, or within about 10 nucleotides, or within about 11 nucleotides, or within about 12 nucleotides, or within about 13 nucleotides, or within about 14 nucleotides, or within about 15 nucleotides, or within about 16 nucleotides, or within about 17 nucleotides, or within about 18 nucleotides, or within about 19 nucleotides, or within about 20 nucleotides, or within about 21 nucleotides, or within about 22 nucleotides, or within about 23 nucleotides,

or within about 24 nucleotides, or within about 25 nucleotides, or within about 26 nucleotides, or within about 27 nucleotides, or within about 28 nucleotides, or within about 29 nucleotides, or within about 30 nucleotides, or within about 40 nucleotides, or within about nucleotides, or within about 50 nucleotides, or within about 60 nucleotides, or within about 70 nucleotides, or within about 80 nucleotides, or within about 90 nucleotides, or within about 100 nucleotides.

As used herein, the term "spatially detecting" is used in its broadest sense to refer to the identification of the presence of a specific target analyte within a specific region of interest in a sample. Spatially detecting can comprise quantifying the amount of a specific target analyte present within a specific region of interest in a sample. Spatially detecting can further comprise quantifying the relative amount of a first target analyte within a specific region of interest in a sample as compared to the amount of at least a second target analyte within a specific region of interest in a sample. Spatially detecting can also comprise quantifying the relative amount of a specific target analyte within a first region of interest in a sample compared to the amount of the same target analyte in at least a second region of interest in the same sample or different sample.

In some aspects of the methods and compositions of the present disclosure, a target analyte can be any molecule within a sample that is to be spatially detected. Target analytes include, but are not limited to, nucleic acid molecules and protein molecules. When the target analyte is a protein, the protein can be referred to as a target protein. When the target analyte is a nucleic acid, the nucleic acid can be referred to as a target nucleic acid. Target nucleic acids can include, but are not limited to, mRNA molecules, micro RNA (miRNA) molecules, tRNA molecules, rRNA molecules, gDNA or any other nucleic acid present within a sample.

In some aspects of the methods and compositions of the present disclosure, the term target binding domain is used in its broadest sense to refer to a portion of a probe of the present disclosure that binds to, either directly or indirectly, a target analyte located in a sample. A target binding domain can comprise nucleic acid, protein, at least one antibody, an aptamer, or any combination thereof. A target binding domain can comprise DNA, RNA or any combination thereof. A target binding domain can comprise any number of modified nucleotides and/or nucleic acid analogues.

In the aspect that the target analyte to be spatially detected is a target protein, a target binding domain can be a protein-target binding domain. A protein-target binding domain can comprise an antibody or antibody fragment that binds to the target protein.

In the aspect that the target analyte to be spatially detected is a target nucleic acid, a target binding domain can be a target nucleic acid-binding region. A target nucleic acid-binding region can comprise a nucleic acid that is complementary to the target nucleic acid to be spatially detected. A target nucleic acid-binding region can comprise a nucleic acid that hybridizes to the target nucleic acid to be detected.

As used herein, the term "hybridize" is used in its broadest sense to mean the formation of a stable nucleic acid duplex. In one aspect, "stable duplex" means that a duplex structure is not destroyed by a stringent wash under conditions such as, for example, a temperature of either about 5° C. below or about 5° C. above the Tm of a strand of the duplex and low monovalent salt concentration, e.g., less than 0.2 M, or less than 0.1 M or salt concentrations known to those of skill in the art. A duplex can be "perfectly matched", such that the polynucleotide and/or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. The term "duplex" comprises, but is not limited to, the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, PNAs, and the like, that can be employed. A duplex can comprise at least one mismatch, wherein the term "mismatch" means that a pair of nucleotides in the duplex fail to undergo Watson-Crick bonding.

As used herein, the term "hybridization conditions," will typically include salt concentrations of less than about 1 M, more usually less than about 500 mM and even more usually less than about 200 mM. Hybridization temperatures can be as low as 5° C., but are typically greater than 22° C., more typically greater than about 30° C., and often in excess of about 37° C. Hybridizations are usually performed under stringent conditions, e.g., conditions under which a probe will specifically hybridize to its target analyte. Stringent conditions are sequence-dependent and are different in different circumstances. Longer fragments can require higher hybridization temperatures for specific hybridization. As other factors can affect the stringency of hybridization, including base composition and length of the complementary strands, presence of organic solvents and extent of base mismatching, the combination of parameters is more important than the absolute measure of any one alone. Certain hybridization conditions will promote the formation of a duplex between the entire length of a target binding domain and the target analyte. Other hybridization conditions will promote the formation of a duplex only along certain portions of the target binding domain.

In some aspects of the methods and compositions of the present disclosure, a probe can comprise a target binding domain directly, or indirectly linked to an identifier oligonucleotide. In the context of a probe, an identifier oligonucleotide is a polynucleotide that comprises a nucleic acid sequence that identifies the target analyte bound to the target binding domain of that probe. That is to say, the identifier oligonucleotide comprises a specific nucleic acid sequence that is a priori assigned to the specific target analyte bound to the target binding to which the identifier oligonucleotide is attached. In a non-limiting example, a probe designated as "probe X" designed to spatially detect "target analyte X" comprises a target binding domain designated "target binding domain X" linked to an identifier oligonucleotide designated "identifier oligonucleotide X". Target binding domain X binds to target analyte X and identifier oligonucleotide X comprises a nucleic acid sequence, designated as "nucleic acid sequence X", which corresponds to target analyte X Thus, if a skilled artisan practicing the methods of the present disclosure were to collect identifier oligonucleotides released from a region of interest in sample and obtain nucleic acid sequence X after sequencing, the skilled artisan would understand that to indicate that target analyte X was present in that region of interest. The amount, or number of sequencing reads, of nucleic acid sequence X can be used to determine the quantify, in absolute or relative terms, the amount of target analyte X within the region of interest.

As used herein, the term "amplification primer binding site" is used in its broadest sense to refer to a nucleic acid sequence that is complementary to, or at least partially complementary to at least one amplification primer, wherein the amplification primer is a short single-stranded or partially single-stranded oligonucleotide that is sufficient to prime DNA and/or RNA synthesis, for example, by PCR.

In some aspects of the methods and compositions of the present disclosure, a target binding domain can be linked to an identifier oligonucleotide by a cleavable linker. Suitable cleavable linkers include, but are not limited to, chemically cleavable linkers (e.g. a linker that is cleaved when exposed to a particular chemical, combination of chemicals or reaction conditions), a photo-cleavable linker (e.g. a linker that is cleaved when exposed to light of a sufficient wavelength or light comprising a sufficient range of wavelengths), or an enzymatically cleavable linker (e.g. a linker that is cleaved by a specific enzyme or class of enzymes). Thus, as used herein the phrase "providing a force to a location of the sample sufficient to release an identifier oligonucleotide" is used in its broadest sense to describe changing the conditions within a certain region of interest in a sample such that, for any probe bound to a target analyte within that region of interest, the linker between the target binding domain of the probe and the identifier oligonucleotide of the probe is cleaved, thereby separating the identifier oligonucleotide from the target binding domain so that the identifier oligonucleotide can be subsequently collected from solution. For example, in aspects wherein a probe comprises a chemically cleavable linker between the target binding domain and the identifier oligonucleotide, providing a force to a location of the sample sufficient to release an identifier oligonucleotide can comprise exposing that location of the sample to the specific chemical, combination of chemicals or reaction conditions that catalyze the cleavage of the linker. In another non-limiting example, in aspects wherein a probe comprises a photo-cleavable linker between the target binding domain and the identifier oligonucleotide, providing a force to a location of the sample sufficient to release an identifier oligonucleotide can comprise exposing/exciting that location of the sample with light of a sufficient wavelength capable of cleaving the photo-cleavable linker. In another non-limiting example, in aspects wherein a probe comprises an enzymatically cleavable linker between the target binding domain and the identifier oligonucleotide, providing a force to a location of the sample sufficient to release an identifier oligonucleotide can comprise exposing that location of the sample to an amount of enzyme sufficient to catalyze the cleavage of the linker.

Providing a force to a location of the sample sufficient to release an identifier oligonucleotide can result in at least about 10%, or at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least about 99% of probes bound to target analytes within that location of the sample to undergo cleavage of the linker connecting the target binding domain and the identifier oligonucleotide.

As would be appreciated by one skilled in the art, the term "unique molecular identifier" or "UMI" refer to short nucleic acid sequences that are used to quantify and reduce quantitative bias caused by nucleic acid amplification prior to sequencing reactions.

In some aspects of the methods and compositions of the present disclosure, an affinity moiety can comprise biotin, avidin, streptavidin, nucleic acid, or any combination thereof.

In some aspects of the methods and compositions of the present disclosure, a probe can comprise at least at least about 5, about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150, at least about 160, at least about 170, at least about 180, at least about 190 or at least about 200 nucleotides.

In some aspects of the methods and compositions of the present disclosure, a target binding domain can comprise at least at least about 5, about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150, at least about 160, at least about 170, at least about 180, at least about 190 or at least about 200 nucleotides.

In some aspects of the methods and compositions of the present disclosure, an identifier oligonucleotide can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150, at least about 160, at least about 170, at least about 180, at least about 190 or at least about 200 nucleotides.

In some aspects of the methods and compositions of the present disclosure, an amplification primer can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150, at least about 160, at least about 170, at least about 180, at least about 190 or at least about 200 nucleotides.

In some aspects of the methods and compositions of the present disclosure, a nucleic acid probe can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150, at least about 160, at least about 170, at least about 180, at least about 190 or at least about 200 nucleotides.

In some aspects of the methods and compositions of the present disclosure, a nucleic acid complementary to a portion of a identifier oligonucleotide can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95 or at least about 100 nucleotides.

In some aspects of the methods and compositions of the present disclosure, a nucleic acid sequence comprising a molecular identifier can comprise at least about 5, or at least about 10 nucleotides, or at least about 15, or at least about

20, or at least about 25, or at least about 30, or at least about 35, or at least about 40, or at least about 45, or at least about 50 nucleotides.

In some aspects of the methods and compositions of the present disclosure, an amplification primer binding site can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35, at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65 or at least about 70 nucleotides.

In some aspects of the methods and compositions of the present disclosure, a flow cell adapter sequence suitable for sequencing can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95 or at least about 100 nucleotides.

In some aspects of the methods and compositions of the present disclosure, a flow cell binding site can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35, at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 90, at least about 95 or at least about 100 nucleotides.

In some aspects of the methods and compositions of the present disclosure, a nucleic acid sequence which identifies the specific location of the tissue sample from which an identifier oligonucleotide was released can comprise at least about 5, or at least about 10 nucleotides, or at least about 15, or at least about 20, or at least about 25, or at least about 30, or at least about 35, or at least about 40, or at least about 45, or at least about 50 nucleotides.

In some aspects of the methods and compositions of the present disclosure, a unique nucleic acid sequence which identifies the target analyte bound to a target binding domain can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35, at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 90, at least about 95 or at least about 100 nucleotides.

In some aspects of the methods and compositions of the present disclosure, a probe, a target binding domain, an identifier oligonucleotide, an amplification primer, a nucleic acid probe, a nucleic acid complementary to a portion of a identifier oligonucleotide, a nucleic acid sequence comprising a molecular identifier, an amplification primer binding site, a flow cell adapter sequence, a flow cell binding site, a nucleic acid sequence which identifies the specific location of the tissue sample from which an identifier oligonucleotide was released, a unique nucleic acid sequence which identifies the target analyte bound to a target binding domain or any combination thereof can comprise at least one natural base, can comprise no natural bases, can comprise at least one modified nucleotide or nucleic acid analog, can comprise no modified nucleotides or nucleic acid analogs, can comprise at least one universal base, can comprise no universal bases, can comprise at least one degenerate base or can comprise no degenerate bases.

In some aspects of the methods and compositions of the present disclosure, a probe, a target binding domain, an identifier oligonucleotide, an amplification primer, a nucleic acid probe, a nucleic acid complementary to a portion of a identifier oligonucleotide, a nucleic acid sequence comprising a molecular identifier, an amplification primer binding site, a flow cell adapter sequence, a flow cell binding site, a nucleic acid sequence which identifies the specific location of the tissue sample from which an identifier oligonucleotide was released, a unique nucleic acid sequence which identifies the target analyte bound to a target binding domain or any combination thereof can comprise any combination natural bases (e.g. 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more natural bases), modified nucleotides or nucleic acid analogs (e.g. 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more modified or analog nucleotides), universal bases (e.g. 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more universal bases), or degenerate bases (e.g. 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more degenerative bases). When present in a combination, the natural bases, modified nucleotides or nucleic acid analogs, universal bases and degenerate bases can be arranged in any order.

The terms "modified nucleotides" or "nucleic acid analogues" include, but are not limited to, locked nucleic acids (LNA), bridged nucleic acids (BNA), propyne-modified nucleic acids, zip nucleic acids (ZNA®), isoguanine and isocytosine. Preferably, the modified nucleotides or nucleic acid analogues are locked nucleic acids (LNAs).

The term "locked nucleic acids (LNA)" as used herein includes, but is not limited to, a modified RNA nucleotide in which the ribose moiety comprises a methylene bridge connecting the 2' oxygen and the 4' carbon. This methylene bridge locks the ribose in the 3'-endo confirmation, also known as the north confirmation, that is found in A-form RNA duplexes. The term inaccessible RNA can be used interchangeably with LNA. The term "bridged nucleic acids (BNA)" as used herein includes, but is not limited to, modified RNA molecules that comprise a five-membered or six-membered bridged structure with a fixed 3'-endo confirmation, also known as the north confirmation. The bridged structure connects the 2' oxygen of the ribose to the 4' carbon of the ribose. Various different bridge structures are possible containing carbon, nitrogen, and hydrogen atoms. The term "propyne-modified nucleic acids" as used herein includes, but is not limited to, pyrimidines, namely cytosine and thymine/uracil, that comprise a propyne modification at the C5 position of the nucleic acid base. The term "zip nucleic acids (ZNA®)" as used herein includes, but is not limited to, oligonucleotides that are conjugated with cationic spermine moieties.

The term "universal base" as used herein includes, but is not limited to, a nucleotide base does not follow Watson-Crick base pair rules but rather can bind to any of the four canonical bases (A, T/U, C, G) located on the target nucleic acid. The term "degenerate base" as used herein includes, but is not limited to, a nucleotide base that does not follow Watson-Crick base pair rules but rather can bind to at least two of the four canonical bases A, T/U, C, G), but not all four. A degenerate base can also be termed a Wobble base; these terms are used interchangeably herein.

As used in this Specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive and covers both "or" and "and".

Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear

from the context, all numerical values provided herein are modified by the term "about."

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although other probes, compositions, methods, and kits similar, or equivalent, to those described herein can be used in the practice of the present disclosure, the preferred materials and methods are described herein. It is to be understood that the terminology used herein is for the purpose of describing particular aspects only, and is not intended to be limiting.

EXAMPLES

Example 1—Two-Ended Adapter Ligation Method for 96 Multiplexed Samples

In this example, a two-ended adapter ligation method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. The nucleic acid adapters used in this experiment were partially double-stranded. The nucleic acid adapters comprised a first strand and a second strand. The first strand comprised a 5' phosphate moiety for ligation. The first strand also comprised a constant nucleic acid sequence to minimize ligation bias (GCGTAGTG), a nucleic acid sequence comprising a unique molecular identifier, a unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a first amplification primer binding site (SEQ ID NO: 2). The second strand comprised a single overhanging thymine nucleotide at the 3' end, a sequence complementary to the constant nucleic acid sequence to minimize ligation bias present in the first strand, a sequence complementary to the unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released present in the first strand and a second amplification primer binding site (SEQ ID NO: 1).

To form the partially double stranded nucleic acid adapters, first strand oligonucleotides and second strand oligonucleotides were combined in equimolar proportion for a final total oligonucleotide concentration of 28 μM in buffer comprising 50 mM NaCl. The oligonucleotide mixture was heated at 95° C. for 2 minutes and cooled at ambient temperature for 30 minutes, thereby annealing the first stand and second strand oligonucleotides together to form the partially double-stranded nucleic acid adapters. Annealed nucleic acid adaptors were diluted to final concentration ranging between 0.02 μM to 0.002 μM in a solution of 10 mM Tris pH 8 and 0.05% Tween20.

Collected identifier oligonucleotides were end repaired and A-tailed using NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs) with a modified protocol. End repair/A-tail master mix was prepared by combining the following: 627.8 μL of PCR-grade H2O, 143.9 μL of NEBNext Ultra II End Prep Reaction Buffer, and 61.7 μL of NEBNext Ultra II End Prep Enzyme Mix. 8.3 μL of end repair/A-tail master mix was added to 4 μL of each sample of identifier oligonucleotides. The reaction was incubated for 30 minutes at 20° C. with a heated lid of >75° C., followed by a second incubation for 30 minutes at 65° C. The repaired/A-tailed identifier oligonucleotide mixtures were then stored at 4° C.

Following end repair and A-tailing, the nucleic acid adaptors were ligated to the repaired/A-tailed identifier oligonucleotides by adding 6.4 μL of NEBNext Ultra II Ligation Master Mix, 0.2 μL of NEBNext Ligation Enhancer, and 1 μL of the nucleic acid adapter dilution to each repaired/A-tailed identifier oligonucleotide mixture. These reactions were incubated for 15 minutes at 20° C. with the heated lid off and subsequently quenched with 1 μL 0.5M EDTA. All of the reactions were then pooled into a single 15 mL conical tube to form a pooled adapter-ligated sample.

The pooled adaptor-ligated sample was purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 350 of AMPure XP beads and 3.15 mL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). AMPure XP bead cleanup was performed with 3.5 mL of diluted AMPure XP beads and eluted in 200 of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. AMPure XP bead cleanup was then repeated with 400 μL of AMPure XP beads and eluted in 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20 to obtain purified adapter-ligated samples.

Following AMPure XP cleanup, PCR reactions with purified adaptor-ligated sample were prepared to amplify the adapter-ligated identifier oligonucleotides. To 6 μL of the purified adapter-ligated sample, 10 μL of NEBNext Ultra II Q5 Master Mix, 0.2 μL of 100 μM forward and reverse primers, and 3.6 μL of PCR-grade H_2O was added. The forward primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the first amplification primer binding site located on the first strand of the nucleic acid adapter. Table 1 provides the sequences of the forward primers used.

TABLE 1

Forward primers for two-ended adapter ligation.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGA GATCTACACGCTCAGATATA GCCTACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	5
AATGATACGGCGACCACCGA GATCTACACGCTCAGAATAG AGGCACACTCTTTAAGACGA CGTCGCTATGGC CTCTCC	6
AATGATACGGCGACCACCGA GATCTACACGCTCAGACCTA TCCTACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	7
AATGATACGGCGACCACCGA GATCTACACGCTCAGAGGCT CTGAACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	8
AATGATACGGCGACCACCGA GATCTACACGCTCAGAAGGC GAAGACACTCTTTAAGACGA CGTCGCTATGGC CTCTCC	9
AATGATACGGCGACCACCGA GATCTACACGCTCAGATAAT CTTAACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	10

TABLE 1-continued

Forward primers for two-ended adapter ligation.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGA GATCTACACGCTCAGACAGG ACGTACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	11
AATGATACGGCGACCACCGA GATCTACACGCTCAGAGTAC TGACACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	12

The reverse primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the second amplification primer binding site located on the second strand of the nucleic acid adapter. Table 2 provides the sequences of the reverse primers used.

TABLE 2

Reverse primers for two-ended adapter ligation

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCGAGTAATGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	13
CAAGCAGAAGACGGCATACGAGATTCTCCGGAGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	14
CAAGCAGAAGACGGCATACGAGATAATGAGCGGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	15
CAAGCAGAAGACGGCATACGAGATGGAATCTCGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	16
CAAGCAGAAGACGGCATACGAGATTTCTGAATGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	17
CAAGCAGAAGACGGCATACGAGATACGAATTCGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	18
CAAGCAGAAGACGGCATACGAGATAGCTTCAGGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	19
CAAGCAGAAGACGGCATACGAGATGCGCATTAGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	20
CAAGCAGAAGACGGCATACGAGATCATAGCCGGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	21
CAAGCAGAAGACGGCATACGAGATTTCGCGGAGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	22
CAAGCAGAAGACGGCATACGAGATGCGCGAGAGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	23
CAAGCAGAAGACGGCATACGAGATCTATCGCTGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT	24

The optimal number of PCR cycles was determined empirically with triplicate PCR reactions. Alternately, the optimal number of PCR cycles could have been determined using real-time/qPCR. The PCR program used comprised the following steps:

(1) 30 seconds at 98° C.
(2) 10 seconds at 98° C.
(3) 1 minute at 65° C.
(4) Repeating steps (2) and (3) nine times
(5) 5 minutes at 65° C.

The amplified products were purified using 18 μL of AMPure XP beads and eluting with 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

The amplified products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The amplified products were also diluted to 15 pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with a custom spike-in primer comprising the nucleotide sequence

(SEQ ID NO: 25)
ACACTCTTTAAGACGACGTCGCTATGGCCTCTCC.

Example 2—One-Ended Adapter Ligation Method for 96 Multiplexed Samples

In this example, a one-ended adapter ligation method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. The nucleic acid adapters used in this experiment were partially double-stranded. The nucleic acid adapters comprised a first strand and a second strand. The first strand comprised a 5' phosphate moiety for ligation. The first strand also comprised a constant nucleic acid sequence to minimize ligation bias (CACTACGC), a nucleic acid sequence comprising a unique molecular identifier, a unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a first amplification primer binding site (SEQ ID NO: 1). The second strand comprised a single overhanging thymine nucleotide at the 3' end, a sequence complementary to the constant nucleic acid sequence to minimize ligation bias present in the first strand and a sequence complementary to the unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released present in the first strand.

To form the partially double stranded nucleic acid adapters, first strand oligonucleotides and second strand oligonucleotides were combined in equimolar proportion for a final total oligonucleotide concentration of 28 μM in 50 mM NaCl. The oligonucleotide mixture was heated to 95° C. for 2 minutes and cooled at ambient temperature for 30 minutes, thereby annealing the first stand and second strand oligonucleotides together to form the partially double-stranded nucleic acid adapters. Annealed nucleic acid adaptors were diluted to a final concentration ranging between 0.02 μM to 0.002 μM in a solution of 10 mM Tris pH 8 and 0.05% Tween20.

Nucleic acid adapters were ligated to the collected identifier oligonucleotides by addition of 10 μL of 2× rapid ligation buffer (Enzymatics), 1 μL of T4 DNA Rapid Ligase (Enzymatics), and 1 μL of annealed nucleic acid adapter dilutions to each sample of collected identifier oligonucleotides. Samples were incubated for 15 minutes at 20° C. with the heated lid off and subsequently quenched with 1 μL 0.5M EDTA. All of the reactions were then pooled into a single 15 mL conical tube to form a pooled adapter-ligated sample.

The pooled adaptor-ligated sample was purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 350 of AMPure XP beads and 3.15 mL of AMPure XP buffer

(2.5M NaCl, 20% PEG8000). AMPure XP bead cleanup was performed with 3.5 mL of diluted AMPure XP beads and eluted in 200 of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. AMPure XP bead cleanup was then repeated with 400 μL of AMPure XP beads and eluted in 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20 to obtain purified adapter-ligated samples.

Following AMPure XP cleanup, PCR reactions with purified adaptor-ligated sample were prepared to amplify the adapter-ligated identifier oligonucleotides. To 6 μL of the purified adapter-ligated sample, 10 μL of NEBNext Ultra II Q5 Master Mix, 0.2 μL of 100 μM forward and reverse primers, and 3.6 μL of PCR-grade H_2O was added. The forward primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the first amplification primer binding site located on the first strand of the nucleic acid adapter. Table 3 provides the sequences of the forward primers used.

TABLE 3

Forward primers for two-ended adapter ligation.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTA CACGCTCAGATATAGCCTACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	5
AATGATACGGCGACCACCGAGATCTAC ACGCTCAGAATAGAGGCACACTCTT TAAGACGACGTCGCTATGGCCTCTCC	6
AATGATACGGCGACCACCGAGATCTAC ACGCTCAGACCTATCCTACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	7
AATGATACGGCGACCACCGAGATCTAC ACGCTCAGAGGCTCTGAACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	8
AATGATACGGCGACCACCGAGATCTAC ACGCTCAGAAGGCGAAGACACTCTT TAAGACGACGTCGCTATGGCCTCTCC	9
AATGATACGGCGACCACCGAGATCTAC ACGCTCAGATAATCTTAACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	10
AATGATACGGCGACCACCGAGATCTAC ACGCTCAGACAGGACGTACACTCTT TAAGACGACGTCGCTATGGCCTCTCC	11
AATGATACGGCGACCACCGAGATCTAC ACGCTCAGAGTACTGACACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	12

The reverse primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the second amplification primer binding site located on the second strand of the nucleic acid adapter. Table 4 provides the sequences of the reverse primers used.

TABLE 4

Reverse primers for two-ended adapter ligation.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCG AGTAATGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	13
CAAGCAGAAGACGGCATACGAGATTC TCCGGAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	14
CAAGCAGAAGACGGCATACGAGATAA TGAGCGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	15
CAAGCAGAAGACGGCATACGAGATGG AATCTCGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	16
CAAGCAGAAGACGGCATACGAGATTT CTGAATGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	17
CAAGCAGAAGACGGCATACGAGATAC GAATTCGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	18
CAAGCAGAAGACGGCATACGAGATAG CTTCAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	19
CAAGCAGAAGACGGCATACGAGATGC GCATTAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	20
CAAGCAGAAGACGGCATACGAGATCA TAGCCGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	21
CAAGCAGAAGACGGCATACGAGATTT CGCGGAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	22
CAAGCAGAAGACGGCATACGAGATGC GCGAGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	23
CAAGCAGAAGACGGCATACGAGATCT ATCGCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	24

The optimal number of PCR cycles was determined empirically with triplicate PCR reactions. Alternately, the optimal number of PCR cycles could have been determined using real-time/qPCR. The PCR program used comprised the following steps:

(1) 30 seconds at 98° C.
(2) 10 seconds at 98° C.
(3) 1 minute at 65° C.
(4) Repeating steps (2) and (3) nine times
(5) 5 minutes at 65° C.

The amplified products were purified using 18 μL of AMPure XP beads and eluting with 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

The amplified products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The amplified products were also diluted to 15 pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with a custom spike-in primer comprising the nucleotide sequence

(SEQ ID NO: 25)
ACACTCTTTAAGACGACGTCGCTATGGCCTCTCC.

Example 3—Templated-Primer Extension Method for 96 Multiplexed Samples

In this example, a templated-primer extension method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. The single stranded nucleic acid templates used in this example comprised a 3' biotin moiety, a region complementary to the unique nucleic acid sequences present in the collected identifier oligonucleotides, a nucleic acid sequence comprising a unique molecular identifier and a second amplification primer binding sequence

(GTGACTGGAGTTCAGACGTGTG

CTCTTCCGATCT, SEQ ID NO: 26).

Table 5 provides the sequences of the single-stranded nucleic acid templates used in this example.

TABLE 5

Single stranded nucleic acid templates for templated-primer extension method

Single-stranded nucleic acid templates	SEQ ID NO
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNTTGAAGCACAC CGTTTTTCTTTCTTCTTTCACGG	27
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNACCCACAGGTT ATACGGGATTATCCGGTTATCCA	28
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNCGACACCGAGT TCGACCGTTATGTTGGTAGGATC	29
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNCGGTGTGTAAG CGTAACGATGTTGGTGTCGCTCT	30
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNCAGACACTGCG ACAACTCACGATCATGACACAGA	31
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNATATTCTGTAC TCAGTGCCTATCCACCTAATAGG	32
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNTTCAGTTATAA TGTGTCCAGCAGAAGCAGGAATT	33
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNGTCCTTTGTTG GGCGGACCGTAATGAGGAATTTG	34
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNGATGAGACTTC TACATGTCCGATGTTTTTGTGCT	35
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNACTCACACATA GTACTGACACGTAAGATAGGATG	36

TABLE 5-continued

Single stranded nucleic acid templates for templated-primer extension method

Single-stranded nucleic acid templates	SEQ ID NO
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNTTACCCTATCT CGTCTATGTACGTCAGGCTGAAT	37
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNATCAACGTAGG GTAAGGTCATATTTTTACCTTAC	38
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNTTCCCTCTTTC TCCGCTTATGGATGAAAGGACAG	39
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNCCTGCACAGTG AGTTTCTTTCACTCTAACTCTCT	40
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNTGTCGCTCTAG TGTGACTTTTCCACCTCGCATCT	41
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNATATCTTTCTC GGGTAAAGATTAGGCGTCCGATA	42
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNCGATTAGCCGT AGACGCAACTCATTGCCGAAGAT	43
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNTGTGAGCATTT CAGTACGAGTGATGCAGATAAAC	44
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNTATAGTTACCA AGTACTATGGGTTGGTGGAAGCC	45
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNCCAATTATACT GTCTGTTATGTTCTCGGATAAGC	46
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNTCAGGTGCTTG TAGGCTCATGATAGGGGTAATGC	47
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNCTCTGCTGTAA TCTCAGCTCCACTTGTTTCTAAG	48
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNGTGCATATTGC AGCTGAGCCAGCTCAATTTGAAG	49
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNCCGTTGATTTA CGCAACAGCGGCTTATATAGCTC	50
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNCATCATCGACA GTTCGCAGCCCTATAACATGATA	51
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNATCGCAGGATG GTACAGCATCATACATGATGAGC	52
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNCTGATAAGTCG TAGGAATGTCGCTTAATACGGAT	53
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNNATGGCGGTTTC GGGTCCTGCACTATTCCTAATAA	54

TABLE 5-continued

Single stranded nucleic acid templates for templated-primer extension method

Single-stranded nucleic acid templates	SEQ ID NO
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNCCAGTACGGGT ACTAATAAGTGTCATATCTATTG	55
GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNNTGTTGGAGAGG TTAGAGGTGAGGAGGCGAAGATA	56

Single stranded nucleic acid templates were ordered from Integrated DNA Technologies, Inc. and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Individual single stranded nucleic acid templates were normalized to a standard concentration and then pooled to be equimolar. The pool of single stranded nucleic acid templates was diluted to 0.83 nM in a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

The collected identifier oligonucleotides were hybridized to the single stranded nucleic acid templates and extended by addition of 10 μL of NEBNext Ultra II Q5 Master Mix (New England Biolabs), 4 μL of the diluted single stranded nucleic acid template pool and 4 μL of H_2O to 2 μL of each sample of identifier oligonucleotides. The following PCR program was used to extend the identifier oligonucleotides:

(1) 30 seconds at 98° C., 10×
(2) 1 minutes at 98° C.,
(3) 1 minutes at 68° C.
(4) 1 minutes at 72° C.
(5) Repeating steps (2)-(4) ten times
(6) 2 minutes at 72° C.

The extension products were stored at 4° C. Magnetic streptavidin beads (MyOne Streptavidin C1 beads, Thermo Fisher Scientific) were washed in 1× Binding and Washing Buffer (5 mM Tris-HCl, 0.5 mM EDTA, 1M NaCl), and 5 μL of streptavidin beads were added to each extension product sample. The extension product samples were incubated with the beads on an orbital mixer for a minimum of 15 minutes. Following incubation, the samples were heated to 95° C. for 3 minutes and transferred to a magnetic plate. Supernatant was extracted immediately after sufficient bead pelleting to yield the purified extension product samples.

The purified extension product samples were amplified by adding to 7.5 μL of each purified extension product sample, 12.5 μL of NEBNext Ultra II Q5 Master Mix, 0.25 μL of 100 μM forward primer, 1 μL of 25 μM reverse primer and 3.8 μL of PCR-grade H_2O. The forward primer comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the first amplification primer binding site located on the identifier oligonucleotide. Table 6 provides the sequences of the forward primers used in this example.

TABLE 6

Forward primers for templated-primer extension method.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACA CGCTCAGATATAGCCTACACTCTTTA AGACGACGTCGCTATGGCCTCTCC	5

TABLE 6-continued

Forward primers for templated-primer extension method.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACA CGCTCAGAATAGAGGCACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	6
AATGATACGGCGACCACCGAGATCTACA CGCTCAGACCTATCCTACACTCTTTA AGACGACGTCGCTATGGCCTCTCC	7
AATGATACGGCGACCACCGAGATCTACA CGCTCAGAGGCTCTGAACACTCTTTA AGACGACGTCGCTATGGCCTCTCC	8
AATGATACGGCGACCACCGAGATCTACA CGCTCAGAAGGCGAAGACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	9
AATGATACGGCGACCACCGAGATCTACA CGCTCAGATAATCTTAACACTCTTTA AGACGACGTCGCTATGGCCTCTCC	10
AATGATACGGCGACCACCGAGATCTACA CGCTCAGACAGGACGTACACTCTTTA AGACGACGTCGCTATGGCCTCTCC	11
AATGATACGGCGACCACCGAGATCTACA CGCTCAGAGTACTGACACACTCTTTA AGACGACGTCGCTATGGCCTCTCC	12

The reverse primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the second amplification primer binding site located single-stranded nucleic acid template. Table 7 provides the sequences of the reverse primers used.

TABLE 7

Reverse primers for templated-primer extension method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATGT CGGTAAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	57
CAAGCAGAAGACGGCATACGAGATAG GTCACTGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	58
CAAGCAGAAGACGGCATACGAGATGA ATCCGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	59
CAAGCAGAAGACGGCATACGAGATGT ACCTTGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	60
CAAGCAGAAGACGGCATACGAGATCA TGAGGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	61
CAAGCAGAAGACGGCATACGAGATTG ACTGACGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	62
CAAGCAGAAGACGGCATACGAGATCG TATTCGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	63

TABLE 7-continued

Reverse primers for templated-primer extension method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCT CCTAGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	64
CAAGCAGAAGACGGCATACGAGATTA GTTGCGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	65
CAAGCAGAAGACGGCATACGAGATGA GATACGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	66
CAAGCAGAAGACGGCATACGAGATAG GTGTACGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	67
CAAGCAGAAGACGGCATACGAGATTA ATGCCGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	68
CAAGCAGAAGACGGCATACGAGATTC AGACGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	69
CAAGCAGAAGACGGCATACGAGATGA TAGGCTGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	70
CAAGCAGAAGACGGCATACGAGATTG GTACAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	71
CAAGCAGAAGACGGCATACGAGATCA AGGTCTGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	72
CAAGCAGAAGACGGCATACGAGATGC TATCCTGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	73
CAAGCAGAAGACGGCATACGAGATATG GAAGGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	74
CAAGCAGAAGACGGCATACGAGATTCA AGGACGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	75
CAAGCAGAAGACGGCATACGAGATGTT ACGCAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	76
CAAGCAGAAGACGGCATACGAGATAGT CTGTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	77
CAAGCAGAAGACGGCATACGAGATGCA CGTAAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	78
CAAGCAGAAGACGGCATACGAGATAAC CTTGGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	79
CAAGCAGAAGACGGCATACGAGATATT GCGTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	80
CAAGCAGAAGACGGCATACGAGATACC TGGAAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	81
CAAGCAGAAGACGGCATACGAGATGGA GATGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	82
CAAGCAGAAGACGGCATACGAGATGTA CTCTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	83
CAAGCAGAAGACGGCATACGAGATGTA ACGACGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	84
CAAGCAGAAGACGGCATACGAGATATT CCTCCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	85
CAAGCAGAAGACGGCATACGAGATGTG TTCCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	86
CAAGCAGAAGACGGCATACGAGATAAG CACTGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	87
CAAGCAGAAGACGGCATACGAGATCTA GCAAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	88
CAAGCAGAAGACGGCATACGAGATTGC TTCCAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	89
CAAGCAGAAGACGGCATACGAGATGCT TAGCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	90
CAAGCAGAAGACGGCATACGAGATAAC CGTTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	91
CAAGCAGAAGACGGCATACGAGATGAC ATTCCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	92
CAAGCAGAAGACGGCATACGAGATAGA CCGTAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	93
CAAGCAGAAGACGGCATACGAGATGAT ACTGGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	94
CAAGCAGAAGACGGCATACGAGATTGC GTAGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	95
CAAGCAGAAGACGGCATACGAGATTCG GTTACGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	96
CAAGCAGAAGACGGCATACGAGATATG ACGTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	97
CAAGCAGAAGACGGCATACGAGATGCT GTAAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	98
CAAGCAGAAGACGGCATACGAGATGCA ATGGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	99

TABLE 7-continued

Reverse primers for templated-primer extension method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATATC TCGCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	100
CAAGCAGAAGACGGCATACGAGATGGC TATTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	101
CAAGCAGAAGACGGCATACGAGATGGT GTCTTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	102
CAAGCAGAAGACGGCATACGAGATTCA ACTGGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	103
CAAGCAGAAGACGGCATACGAGATCTT CACCAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	104
CAAGCAGAAGACGGCATACGAGATACG GTCTTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	105
CAAGCAGAAGACGGCATACGAGATTCT CGCAAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	106
CAAGCAGAAGACGGCATACGAGATGGA ATTGCGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	107
CAAGCAGAAGACGGCATACGAGATACG GATTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	108
CAAGCAGAAGACGGCATACGAGATTTA AGCGGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	109
CAAGCAGAAGACGGCATACGAGATTGC AGGTAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	110
CAAGCAGAAGACGGCATACGAGATCAA TCGACGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	111
CAAGCAGAAGACGGCATACGAGATGTG CCATAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	112
CAAGCAGAAGACGGCATACGAGATTGT TCGAGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	113
CAAGCAGAAGACGGCATACGAGATTGG AGTTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	114
CAAGCAGAAGACGGCATACGAGATACG ATGACGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	115
CAAGCAGAAGACGGCATACGAGATTGA TGTCCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	116
CAAGCAGAAGACGGCATACGAGATTGA ACCTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	117

TABLE 7-continued

Reverse primers for templated-primer extension method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCTT CGTTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	118
CAAGCAGAAGACGGCATACGAGATCTT CTGAGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	119
CAAGCAGAAGACGGCATACGAGATTGC TCATGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	120
CAAGCAGAAGACGGCATACGAGATAGT TCGTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	121
CAAGCAGAAGACGGCATACGAGATTAG CGTCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	122
CAAGCAGAAGACGGCATACGAGATGGC GTTATGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	123
CAAGCAGAAGACGGCATACGAGATGGT GATTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	124
CAAGCAGAAGACGGCATACGAGATAAC TTGCCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	125
CAAGCAGAAGACGGCATACGAGATGCA AGATCGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	126
CAAGCAGAAGACGGCATACGAGATTCG CATTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	127
CAAGCAGAAGACGGCATACGAGATTGT ACACCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	128
CAAGCAGAAGACGGCATACGAGATAGC TCCTAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	129
CAAGCAGAAGACGGCATACGAGATGCA ATTCGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	130
CAAGCAGAAGACGGCATACGAGATCTT AGGACGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	131
CAAGCAGAAGACGGCATACGAGATGTC CTAAGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	132
CAAGCAGAAGACGGCATACGAGATAAC GTGGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	133
CAAGCAGAAGACGGCATACGAGATCTG TGTTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	134
CAAGCAGAAGACGGCATACGAGATGTT AAGGCGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	135

TABLE 7-continued

Reverse primers for templated-primer extension method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCAC CTTACGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	136
CAAGCAGAAGACGGCATACGAGATTGG TAGCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	137
CAAGCAGAAGACGGCATACGAGATCAG TGAAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	138
CAAGCAGAAGACGGCATACGAGATGTT CAACCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	139
CAAGCAGAAGACGGCATACGAGATTGG CTATCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	140
CAAGCAGAAGACGGCATACGAGATCTG GAGTAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	141
CAAGCAGAAGACGGCATACGAGATTCT CTTCCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	142
CAAGCAGAAGACGGCATACGAGATTCT AACGCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	143
CAAGCAGAAGACGGCATACGAGATGGT CAGATGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	144
CAAGCAGAAGACGGCATACGAGATCTC TGGTTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	145
CAAGCAGAAGACGGCATACGAGATTGT GGTACGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	146
CAAGCAGAAGACGGCATACGAGATCCT ATACCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	147
CAAGCAGAAGACGGCATACGAGATTTC TCTCGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	148
CAAGCAGAAGACGGCATACGAGATGTA TGCTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	149
CAAGCAGAAGACGGCATACGAGATAAG TCGAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	150
CAAGCAGAAGACGGCATACGAGATAAC CGAAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	151
CAAGCAGAAGACGGCATACGAGATTGT TGTGGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	152

The PCR program used to amplify the purified extension products comprised the following steps:

(1) 30 seconds at 98° C.

(2) 10 seconds at 98° C.

(3) 30 seconds at 65° C.

(4) 30 seconds at 72° C.

(5) Repeat steps (2)-(4) eighteen times

(6) 2 minutes at 72° C.

The amplified extension products were stored at 4° C. 4 μL of each PCR reaction were combined into four pools, for 24 samples per pool.

The pooled PCR reactions were purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 100 μL of AMPure XP beads and 400 μL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). Purification was performed with 76.8 μL of diluted AMPure XP beads and eluted in 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. Beads were retained and cleanup process was repeated with 24 μL of AMPure XP buffer and eluted in 20 μL of buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

The purified PCR products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The purified PCR products were also diluted to 15 pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with a custom spike-in primer comprising the nucleotide sequence

(SEQ ID NO: 25)
ACACTCTTTAAGACGACGTCGCTATGGCCTCTCC.

Example 4—Long Probe Hybridization Method for 96 Multiplexed Samples

In this example, a long probe hybridization method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. In this example, the first nucleic acid probe comprises a 5' phosphate moiety, a nucleic acid sequence complementary to a portion of the identifier oligonucleotide, a first amplification primer binding site comprising an i7 sequence (SEQ ID NO: 1), unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a P7 flow cell adapter sequence (SEQ ID NO: 4). The second nucleic acid probe comprises a nucleic acid sequence complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier, a second amplification primer binding site comprising and i5 sequence (SEQ ID NO: 2) and a P5 flow cell adapter sequence (SEQ ID NO: 3).

The first and second nucleic acid probes were ordered from Integrated DNA Technologies, Inc. and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Individual nucleic acid probes were normalized to a standard concentration, pooled to be equimolar, and diluted to 0.83 nM in a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. The nucleic acid probes and the identifier oligonucleotides were hybridized by combining 0.5 μL of diluted nucleic acid probe pool with 2 μL of a mixture of identifier oligonucleotides collected from a sample solution in a buffer comprising 50 mM NaCl. This mixture was heated for 2 minutes at 95° C. and cooled for 30 minutes at ambient temperature to yield an annealed identifier oligonucleotide-nucleic acid probe mixture.

In the case in which the first and the second nucleic acid probes hybridized to the identifier oligonucleotide such that

the first and the second nucleic acid probes were not adjacent and were not overlapping, a gap extension reaction was performed. To 2.5 μL of each annealed identifier oligonucleotide-nucleic acid probe mixture, 3.8 μL of NEBNext Ultra II Q5 Master mix and 1.3 μL of PCR-grade H_2O was added. The mixture was then subjected to the following Gap extension temperature cycle:

(1) 30 seconds at 98° C.
(2) 1 minute at 98° C.
(3) 1 minute at 68° C.
(4) 1 minute at 72° C.,
(5) Repeat steps (2)-(4) ten times
(6) 2 minutes at 72° C.

The gap extension products were then stored at 4° C. The first and second nucleic acid probes were then ligated together by adding to 1 μL of the gap extension product, 10 μL of 2× rapid ligation buffer (Enzymatics), 1 μL of T4 DNA Rapid Ligase (Enzymatics), and 8 μL of PCR-grade H_2O. These ligation reactions were incubated for 15 minutes 20° C., subsequently quenched with 1 μL 0.5M EDTA, and pooled into a single 15 mL conical tube.

In the case in which the first and the second nucleic acid probes hybridized to the identifier oligonucleotide such that the first and the second nucleic acid probes were adjacent and were not overlapping, nick repair ligation reaction was performed. To 2.5 μL of each annealed identifier oligonucleotide-nucleic acid probe mixture, 10 μL of 2× rapid ligation buffer (Enzymatics), 1 μL of T4 DNA Rapid Ligase (Enzymatics), and 1 μL of PCR-grade H_2O was added. These ligation reactions were incubated for 15 minutes at 20° C., subsequently quenched with 1 μL of 0.5M EDTA, and pooled into a single 15 mL conical tube.

The pools of quenched ligation reactions were then purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 350 μL of AMPure XP beads and 3.15 mL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). The purification was performed with 3.5 mL of diluted AMPure XP beads and eluted in 200 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. The AMPure XP bead cleanup was then repeated with 400 μL of AMPure XP beads and eluted in 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

To amplify the purified ligation products, PCR reactions with purified ligation products and primers were prepared. To 6 μL of purified ligation product, 10 μL of NEBNext Ultra II Q5 Master Mix, 0.2 μL of 100 μM forward primer (CAAGCAGAAGACGGCATACGA, SEQ ID NO: 153) and reverse primer (AATGATACGGCGACCACCGA, SEQ ID NO: 154) and 3.6 of PCR-grade H_2O was added. The PCR program used to amplify the purified extension products comprised the following steps:

(1) 30 seconds at 98° C.
(2) 10 seconds at 98° C.
(3) 30 seconds at 65° C.
(4) 30 seconds at 72° C.
(5) Repeat steps (2)-(4) eighteen times
(6) 2 minutes at 72° C.

The amplified products were stored at 4° C. 4 μL of each PCR reaction were combined into six pools, for 16 samples per pool. The amplified products were further purified using an AMPure XP bead cleanup with 64 μL of AMPure XP beads and eluting with 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

The purified amplified products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The purified amplified products were also diluted to 15 pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with either standard sequencing primers or a custom spike-in Read1 primer (SEQ ID NO: 25).

Example 5—Short Probe Hybridization Method for 96 Multiplexed Samples

In this example, a short probe hybridization method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. In this example, the first nucleic acid probe comprises a 5' phosphate moiety, a nucleic acid sequence complementary to a portion of the identifier oligonucleotide, a first amplification primer binding site comprising an i7 sequence (SEQ ID NO: 1) and a unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. The second nucleic acid probe comprises a nucleic acid sequence complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier and a second amplification primer binding site comprising an i5 sequence (SEQ ID NO: 2).

The first and second nucleic acid probes were ordered from Integrated DNA Technologies, Inc. and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Individual nucleic acid probes were normalized to a standard concentration, pooled to be equimolar, and diluted to 0.83 nM in a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. The nucleic acid probes and the identifier oligonucleotides were hybridized by combining 0.5 μL of diluted nucleic acid probe pool with 2 μL of a mixture of identifier oligonucleotides collected from a sample solution in a buffer comprising 50 mM NaCl. This mixture was heated for 2 minutes at 95° C. and cooled for 30 minutes at ambient temperature to yield an annealed identifier oligonucleotide-nucleic acid probe mixture.

In the case in which the first and the second nucleic acid probes hybridized to the identifier oligonucleotide such that the first and the second nucleic acid probes were not adjacent and were not overlapping, a gap extension reaction was performed. To 2.5 μL of each annealed identifier oligonucleotide-nucleic acid probe mixture, 3.8 μL of NEBNext Ultra II Q5 Master mix and 1.3 μL of PCR-grade H_2O was added. The mixture was then subjected to the following Gap extension temperature cycle:

(1) 30 seconds at 98° C.
(2) 1 minute at 98° C.
(3) 1 minute at 68° C.
(4) 1 minute at 72° C.,
(5) Repeat steps (2)-(4) ten times
(6) 2 minutes at 72° C.

The gap extension products were then stored at 4° C. The first and second nucleic acid probes were then ligated together by adding to 1 μL of the gap extension product, 10 μL of 2× rapid ligation buffer (Enzymatics), 1 μL of T4 DNA Rapid Ligase (Enzymatics), and 8 μL of PCR-grade H_2O. These ligation reactions were incubated 15 min 20° C., quenched with 1 μL 0.5M EDTA, and pooled into a single 15 mL conical tube.

In the case in which the first and the second nucleic acid probes hybridized to the identifier oligonucleotide such that the first and the second nucleic acid probes were adjacent and were not overlapping, nick repair ligation reaction was performed. To 2.5 μL of each annealed identifier oligonucle-

otide-nucleic acid probe mixture, 10 μL of 2× rapid ligation buffer (Enzymatics), 1 μL of T4 DNA Rapid Ligase (Enzymatics), and 1 μL of PCR-grade H_2O was added. These ligation reactions were incubated for 15 minutes at 20° C., subsequently quenched with 1 μL of 0.5M EDTA, and pooled into a single 15 mL conical tube.

The pools of quenched ligation reactions were then purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 350 μL of AMPure XP beads and 3.15 mL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). The purification was performed with 3.5 mL of diluted AMPure XP beads and eluted in 200 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. The AMPure XP bead cleanup was then repeated with 400 μL of AMPure XP beads and eluted in 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

To amplify the purified ligation products, PCR reactions with purified ligation products and primers were prepared. To 6 μL of purified ligation product, 10 μL of NEBNext Ultra II Q5 Master Mix, 0.2 μL of 100 μM forward primer and reverse primer and 3.6 μL of PCR-grade H_2O was added. The forward primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the first amplification primer binding site located on the first strand of the nucleic acid adapter. Table 8 provides the sequences of the forward primers used.

TABLE 8

Forward primers for short probe hybridization method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCG AGTAATGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	13
CAAGCAGAAGACGGCATACGAGATTCT CCGGAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	14
CAAGCAGAAGACGGCATACGAGATAAT GAGCGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	15
CAAGCAGAAGACGGCATACGAGATGGA ATCTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	16
CAAGCAGAAGACGGCATACGAGATTTC TGAATGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	17
CAAGCAGAAGACGGCATACGAGATACG AATTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	18
CAAGCAGAAGACGGCATACGAGATAGC TTCAGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	19
CAAGCAGAAGACGGCATACGAGATGCG CATTAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	20
CAAGCAGAAGACGGCATACGAGATCAT AGCCGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	21

TABLE 8-continued

Forward primers for short probe hybridization method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATTTC GCGGAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	22
CAAGCAGAAGACGGCATACGAGATGCG CGAGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	23
CAAGCAGAAGACGGCATACGAGATCTA TCGCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	24

The reverse primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the second amplification primer binding site located on the second strand of the nucleic acid adapter. Table 9 provides the sequences of the reverse primers used.

TABLE 9

Reverse primers for short probe hybridization method.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACACT ATAGCCTACACTCTTTCCCTACACGACGCT CTTCCGATCT	155
AATGATACGGCGACCACCGAGATCTACACA TAGAGGCACACTCTTTCCCTACACGACGCT CTTCCGATCT	156
AATGATACGGCGACCACCGAGATCTACACC CTATCCTACACTCTTTCCCTACACGACGCT CTTCCGATCT	157
AATGATACGGCGACCACCGAGATCTACACG GCTCTGAACACTCTTTCCCTACACGACGCT CTTCCGATCT	158
AATGATACGGCGACCACCGAGATCTACACA GGCGAAGACACTCTTTCCCTACACGACGCT CTTCCGATCT	159
AATGATACGGCGACCACCGAGATCTACACT AATCTTAACACTCTTTCCCTACACGACGCT CTTCCGATCT	160
AATGATACGGCGACCACCGAGATCTACACC AGGACGTACACTCTTTCCCTACACGACGCT CTTCCGATCT	161
AATGATACGGCGACCACCGAGATCTACACG TACTGACACACTCTTTCCCTACACGACGCT CTTCCGATCT	162

The PCR program used to amplify the purified extension products comprised the following steps:

(1) 30 seconds at 98° C.
(2) 10 seconds at 98° C.
(3) 30 seconds at 65° C.
(4) 30 seconds at 72° C.
(5) Repeat steps (2)-(4) eighteen times
(6) 2 minutes at 72° C.

The amplified products were stored at 4° C. 4 μL of each PCR reaction were combined into six pools, for 16 samples

per pool. The amplified products were further purified using an AMPure XP bead cleanup with 64 μL of AMPure XP beads and eluting with 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

The purified amplified products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The purified amplified products were also diluted to 15 pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with either standard sequencing primers or a custom spike-in Read1 primer (SEQ ID NO: 25).

Example 6—Direct PCR Method for 96 Multiplexed Samples

In this example, a direct PCR method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. In this example, 8 species of forward amplification primers and 12 species of reverse amplification primers were used. The forward primers comprised a P5 flow cell adapter (SEQ ID NO: 3), a nucleic acid sequence comprising a unique molecular identifier and a region complementary to a first amplification primer binding site present on the identifier oligonucleotide. Table 10 provides the sequences of the forward amplification primers used.

TABLE 10

Forward amplification primers for direct PCR method.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACACG CTCAGATATAGCCTACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	5
AATGATACGGCGACCACCGAGATCTACACG CTCAGAATAGAGGCACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	6
AATGATACGGCGACCACCGAGATCTACACG CTCAGACCTATCCTACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	7
AATGATACGGCGACCACCGAGATCTACACG CTCAGAGGCTCTGAACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	8
AATGATACGGCGACCACCGAGATCTACACG CTCAGAAGGCGAAGACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	9
AATGATACGGCGACCACCGAGATCTACACG CTCAGATAATCTTAACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	10
AATGATACGGCGACCACCGAGATCTACACG CTCAGACAGGACGTACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	11
AATGATACGGCGACCACCGAGATCTACACG CTCAGAGTACTGACACACTCTTTAAGACGA CGTCGCTATGGCCTCTCC	12

The reverse primers comprised a P7 flow cell adapter (SEQ ID NO: 4), a nucleic acid sequence comprising a unique molecular identifier and a region complementary to a second amplification primer binding site present on the identifier oligonucleotide. Table 11 provides the sequences of the reverse amplification primers used.

TABLE 11

Reverse amplification primers for direct PCR method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCGTGAT GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNAACGGACAGGAT GCAGCAAAAT	163
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CAAGCAGAAGACGGCATACGAGATGTAGCC GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNAACGGACAGGAT GCAGCAAAAT	173
CAAGCAGAAGACGGCATACGAGATTACAAG GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNNNNNNNNNNNAACGGACAGGAT GCAGCAAAAT	174

In the case of 8 forward amplification primers and 12 reverse amplification primers, when the unique molecular identifiers from a pair of forward and reverse primers are combined, a total of 96 unique combinations can be obtained, allowing for the multiplexing of 96 samples.

To amplify the collected identifier oligonucleotides for sequencing, PCR reactions with collected identifier oligonucleotides and forward and reverse amplification primers were prepared on a 96-well plate with 2 μL of each identifier

oligonucleotide sample, 10 μL of NEBNext Ultra II Q5 Master Mix, 2 μL of 10 μM forward amplification primer, 2 μL of reverse amplification primer, and 4 μL of PCR-grade H_2O. Each well in the 96-well plate contained an identifier oligonucleotide sample and a unique combination of forward and reverse amplification primers. The PCR program used comprised the following steps:

(1) 30 seconds at 98° C.
(2) 10 seconds at 98° C.
(3) 30 seconds at 65° C.
(4) 30 seconds at 72° C.
(5) Repeat steps (2)-(4) six to ten times
(6) 2 minutes at 72° C.

The amplified products were stored at 4° C. 10 μL of each PCR reaction was combined into a single 15 mL conical tube.

The pooled PCR reactions were purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 115.2 μL of AMPure XP beads and 1036.8 μL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). Purification was performed with 1152 μL of diluted AMPure XP beads and eluted in 60 μL of a buffer comprising 10 mM Tris pH 8. The purification process was repeated with 60 μL of AMPure XP beads and eluted in 70 μL of a buffer comprising 10 mM Tris pH 8.

Following AMPure XP cleanup, PCR reactions with universal primers were prepared with 9 μL of pooled direct PCR product, 15 μL of NEBNext Ultra II Q5 Master Mix, 3 μL of 10 μM universal P7 primer (SEQ ID NO: 153) and 2 μL of 10 μM universal P5 primer (SEQ ID NO: 154). The PCR program used was:

(1) 30 seconds at 98° C.,
(2) 10 seconds at 98° C.
(3) 30 seconds at 65° C.
(4) 30 seconds at 72° C.
(5) Repeat steps (2)-(4) 15 to 24 times
(6) 2 minutes at 72° C.

Two rounds of AMPure XP bead cleanup was performed. The first round was performed with 30 μL of beads and eluted with 20 μL of a buffer comprising 10 mM Tris pH 8 and the second round was performed with 20 μL beads and eluted with 11 μL of a buffer comprising 10 mM Tris pH 8.

These purified PCR products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics). The purified PCR products were also diluted for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with a custom spike-in primer (SEQ ID NO: 25).

Example 7—Spatially Detecting Target Analytes in a FFPE Sample

The methods of the present invention were used to spatially detect a plurality of different target analytes, including target proteins and target RNAs, in a sample of inflamed human tonsil tissue FFPE section.

In one experiment, 30 different target proteins were spatially detected using the methods of the present disclosure in two serial sections cut from the inflamed human tonsil tissue FFPE section. The 30 target proteins are put forth in Table 12. The 30 target proteins included IgG Rabbit isotype and IgG Mouse isotype as negative controls, as these target proteins should not have been present in the inflamed human tonsil sample and therefore should not have been detected.

TABLE 12

Target Proteins

Target Protein	Target Protein
AKT	FOXP3
B7-H3	GZMB
Bcl-2	Histone H3
Beta-2-microglobulin	Ki67
Beta-catenin	CD20
CD14	P-AKT
CD19	PanCK
CD3	PD1
CD4	PD-L1
CD44	S6
CD45	STAT3
CD45RO	P-STAT3
CD56	VISTA
CD68	IgG Rabbit isotype (negative control)
CD8A	IgG Mouse isotype (control)

To spatially detect the 30 target proteins, 30 different probes of the present disclosure were used. Each probe comprised a target binding domain comprising an antibody that specifically binds to one of the 30 target proteins in Table 12. The two serial sections were contacted with a plurality of the 30 different probes. Ninety-six regions of interest (ROI) were then identified. For each ROI, the ROI was illuminated with UV light to release the identifier oligonucleotides from the probes bound within the ROI. The released identifier oligonucleotides were then collected and identified using a short probe hybridization method of the present disclosure, thereby spatially detecting the 30 target proteins in the two serial sections. As shown in FIGS. **21**A-**21**D, the number of reads per target protein in each ROI for the two serial sections were well correlated, demonstrating that the method yields reproducible results.

In a second experiment, 20 different target RNAs were spatially detected using the methods of the present disclosure in two different serial sections cut from the inflamed human tonsil tissue FFPE section. The 20 different target RNAs are put forth in Table 13. The 20 target RNAs included 6 negative controls (Negative Probe) that should not have been detected in the sample.

TABLE 13

Target RNAs

Target RNA	Target RNA	Target RNA
CD3E	CD40	CTLA4
CD3G	CD45	GAPDH
CD4	CD74	KRT13
CD20	CD79A	PD1
PSA	RP56	Negative Probe #1
Negative Probe #2	Negative Probe #3	Negative Probe #4
Negative Probe #5	Negative Probe #6	

To spatially detect the 20 Target RNAs, 20 different probes of the present disclosure were used. Each probe comprised a target binding domain comprising a nucleic acid sequence complementary to at least one portion of one of the 20 target RNAs. The two serial sections were contacted with a plurality of the 20 different probes. Ninety-six regions of interest (ROI) were then identified. For each ROI, the ROI was illuminated with UV light to release the identifier oligonucleotides from the probes bound within the ROI. The released identifier oligonucleotides were then collected and identified using a direct PCR method of the present disclosure, thereby spatially detecting the 20 target

RNAs in the two serial sections. As shown in FIGS. **22**A-**22**D, the number of reads per target RNA in each ROI for the two serial sections were well correlated, demonstrating that the method yields reproducible results.

Example 8—Spatially Detecting Target Proteins in a Fluorescently Stained FFPE Sample

In another experiment, a 5 μm FFPE section of inflamed human tonsil tissue was stained with 4 fluorescent visualization markers: (1) CD3E, a T-cell marker; (2) PanCK, an epithelial cell marker; (3) Ki-67, a proliferation marker; and (4) SYTO83, a DNA stain, as shown in the left panel of FIG. **23**. The stained FFPE section was then contacted with the probes directed against 30 target proteins, as described in Example 7. As shown in the left panel FIG. **23**, **96** regions of interest (ROIs) were selected. Each ROI was a circle with a 500 μm diameter. For each ROI, the ROI was illuminated with UV light to release the identifier oligonucleotides from the probes bound within the ROI. The released identifier oligonucleotides were then collected and identified using a short probe hybridization method of the present disclosure, thereby spatially detecting the 30 target proteins in the FFPE section. As shown in the right panel of FIG. **23**, PanCK, CD3E and Ki67 were spatially detected in ROIs that correlated with their fluorescent visualization markers. Thus, the results generated by the methods of the present disclosure correlate with the results generated using established immunohistochemical method.

Example 9—Spatially Detecting Target RNAs in a FFPE Sample

In another experiment, a 5 μm section from an inflamed human tonsil tissue FFPE block was contacted with probes directed against 20 target RNAs, as described in Example 7. 96 regions of interest (ROIs) were then selected. Each ROI was a circle with a 500 μm diameter. For each ROI, the ROI was illuminated with UV light to release the identifier oligonucleotides from the probes bound within the ROI. The released identifier oligonucleotides were then collected and identified using a direct PCR method of the present disclosure, thereby spatially detecting the 20 target RNAs in the two serial sections. The total RNA from a 20 μm section from the same inflamed human tonsil tissue FFPE block was then isolated. The total RNA was analyzed using the NanoString nCounter® system. FIG. **24** shows that the average number of counts for 11 different RNA targets recorded using the methods of the present disclosure were well correlated with the average number of counts for the same 11 different RNA targets recorded using the nCounter® system. Thus, the results generated using the methods of the present disclosure correlate with the results generated using established direct detection methods.

Example 10—Spatially Detecting Target RNAs in Specific Sub-Regions of an ROI

In another experiment, a 5 μm section from an inflamed human tonsil tissue FFPE block was contacted with probes directed against 30 target proteins, as described in Example 7. The same 5 μm section was also stained with 4 fluorescent visualization markers: (1) CD3E, a T-cell marker; (2) PanCK, an epithelial cell marker; (3) Ki-67, a proliferation marker; and (4) SYTO83, a DNA stain. As shown in FIG. **25**, **48** regions of interest (ROIs) were identified. For each ROI, two sub-regions were then identified based on the fluorescent staining. Areas of an ROI that were fluorescently stained positive for PanCK (PanCK+) were designated a "tumor" sub-region and the areas of an ROI that lacked PanCK fluorescent staining were designated a "micro-environment" sub-region, as shown in FIG. **25**. For each ROI, the tumor sub-region and the micro-environment sub-region were separately illuminated with UV light to release the identifier oligonucleotides from the probes bound within each sub-region by creating a custom mask based on the intensity of PanCK fluorescent staining. The released identifier oligonucleotides were also separately collected. The collected identifier oligonucleotides were then analyzed using the short-probe hybridization method of the present disclosure and the NanoString nCounter® system. As shown in the bottom panel of FIG. **25**, the results using the NanoString nCounter® system and the short-probe hybridization method of the present disclosure were well correlated. Furthermore, in the tumor sub-regions, PanCK was detected at a significantly higher level as compared to the micro-environment sub-regions. Thus, the spatial detection results provided by the methods of the present disclosure are consistent with established fluorescent immunohistochemical methods and allows for the spatial detection within highly specific regions of a sample.

Example 11—96-Plex Human Immuno-Oncology Panel

A 96-plex human immuno-oncology panel was designed for use in the direct-PCR methods of the present invention. The panel comprised a plurality of probes that could be used to spatially detect 96 different human target RNAs using the direct-PCR methods of the present disclosure. The 96 target RNAs are shown in Table 14.

TABLE 14

Target RNAs

Target	Target	Target	Target	Target	Target	Target	Target
AKT1	CD3E	CEACAM8	FOXP3	IFNG	KRT1	NCAM1	SOD2
ARG1	CD4	CMKLR1	GZMB	IFNGR1	KRT10	NKG7	SOX10
B2M	CD40	CSF1R	H3F3A	IL10	KRT14	NT5E	STAT1
BATF3	CD4OLG	CTLA4	HAVCR2	IL12B	KRT17	PDCD1	STAT2
BCL2	CD44	CTNNB1	HIF1A	IL15	KRT18	PDCD1LG2	STAT3
BCL2L1	CD47	CXCL10	HLA_DQA1	IL1B	KRT19	PECAM1	TBX21
CCL5	CD68	CXCL9	HLA DRB	IL6	KRT6A	PMEL	TIGIT
CCND1	CD74	CSCR6	HLA _E	ITGAM	KRT7	PSMB10	TNF
CD14	CD86	DKK2	ICAM1	ITGAV	LAG3	PTEN	TNFRSF9
CD27	CD8A	EPCAM	ICOSLG	ITGAX	LY6E	PRTPRC	TNFSF4
CD274	CEACAM1	FAS	IDO1	ITGB2	MKI67	RPS6	VEGFA
CD276	CEACAM6	FASLG	IFNAR1	ITGB8	MS4A1	SIOOB	VSIR

In total, the panel comprised 928 different probes. Each of the probes comprised an identifier oligonucleotide comprising a first amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier, a unique nucleic acid sequence which identified the target RNA bound to the target binding domain and a second amplification primer binding site. FIG. **26** shows a schematic of the probes used in the panel. For each of the 96 target RNAs, there was at least one probe within the 928 probe set comprising a target binding domain that directly or indirectly hybridized to that target RNA. For most of the 96 target RNAs, there were 10 different probes that directly or indirectly hybridized to the specific target RNA. These 10 different probes directly or indirectly hybridized to different locations on the target RNA to create a "tiling" effect, as shown in the top panel of FIG. **27**. Tiling multiple probes onto a target RNA means that each target RNA will be individually detected multiple times, increasing the overall accuracy of the measurement. For example, as shown in the bottom panel of FIG. **27**, in the case where 10 probes are tiled onto a single target RNA, one of the probes may be incorrectly detected too many times (outlier high count probe), while another probe may be incorrectly detected too few times (outlier low count probe). However, the other 8 probes may be detected at a similar level, indicating that the two outliers should be discarded during analysis and the signals from the 8 probes used to generate a more accurate measurement of the abundance of the target RNA.

The set of 928 probes also comprised 80 negative control probes. Each of the 80 negative control probes comprised a target binding domain comprising a scrambled, non-specific nucleic acid sequence that was designed using guidelines from the External RNA Controls Consortium such that the target binding domain should not be complementary to RNA molecules present within a human sample. Thus, these 80 negative control probes should not be detected during analysis.

The 96-plex human immune-oncology panel was used to analyze a tissue microarray. The tissue microarray comprised FFPE samples of 22 common human cell lines, including normal and cancerous cell types. Some of the cell lines are shown in Table 15.

TABLE 15

Cell lines			
Cell Line	Cell Line	Cell Line	Cell Line
CCRF-CEM	DAUDI	H596	H2228
HT29	HUT78	HUH7	JURKAT
M14	MDA-MB-468	MOLT4	RAJI
SKBR3	SUDHL1	SUDHL4	

The tissue microarray also comprised one mouse cell line (3T3) as a negative control. Each of the FFPE samples on the microarray was contacted with a plurality of the 928 different probes in the immuno-onocology panel. As shown in FIG. **28**, for each of the FFPE samples, at least three circular regions of interest (ROIs) with a diameter of 300 μm were selected. As a negative control, ROIs were also selected on regions of the microarray that did not comprise a FFPE sample (glass negative control). For each ROI, the ROI was illuminated with UV light to release the identifier oligonucleotides from the probes bound within the ROI. The released identifier oligonucleotides were then collected and identified using a direct PCR method of the present disclosure thereby spatially detecting the 96 target RNAs in each of the FFPE samples on the tissue microarray.

FIG. **29** shows that a sufficient read depth was achieved using a MiSeq v3 flowcell. The top panel of FIG. **30** shows that none of the target RNAs were spatially detected for the glass negative control ROIs. Likewise, the bottom panel of FIG. **30** shows that nearly none of the target RNAs were detected in the negative control mouse 3T3 FFPE sample. Conversely, as shown in FIGS. **31**, **33** and **34**, specific target RNAs were successfully detected in the HEK293 (human embryonic kidney) FFPE sample and the Jurkat (human T-cell lymphocyte) FFPE sample. FIGS. **31**, **33** and **34** show that clusters of "tiled" probes were detected for particular target RNAs, including AKT1, B2M, CD3E, HIF1A, PTEN, RPS6, STAT1, STAT2, STAT3, VEGF, PTPRC (CD45), and KRT1/10/18/19. These results indicated that there are certain target RNAs that are differentially transcribed in the two different cells lines. The results of this experiment were also verified using the NanoString nCounter system to identify the collected identifier oligonucleotides. As shown in FIG. **27**, the results using the direct-PCR method of the present disclosure were consistent with the results obtained using the NanoString nCounter system.

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<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Amplification primer binding site

<400> SEQUENCE: 26

gtgactggag ttcagacgtg tgctcttccg atct 34

<210> SEQ ID NO 27
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P01_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 27

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnt tgaagcacac 60

cgtttttctt tcttctttca cgg 83

<210> SEQ ID NO 28
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P03_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 28

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnna cccacaggtt 60

atacgggatt atccggttat cca 83

<210> SEQ ID NO 29
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P04_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 29

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnc gacaccgagt 60

tcgaccgtta tgttggtagg atc 83

<210> SEQ ID NO 30
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P05_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)

<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 30

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnc ggtgtgtaag 60

cgtaacgatg ttggtgtcgc tct 83

<210> SEQ ID NO 31
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P06_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 31

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnc agacactgcg 60

acaactcacg atcatgacac aga 83

<210> SEQ ID NO 32
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P07_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 32

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnna tattctgtac 60

tcagtgccta tccacctaat agg 83

<210> SEQ ID NO 33
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P09_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 33

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnt tcagttataa 60

tgtgtccagc agaagcagga att 83

<210> SEQ ID NO 34
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P11_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 34

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnng tcctttgttg 60

ggcggaccgt aatgaggaat ttg 83

<210> SEQ ID NO 35
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P13_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 35

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnng atgagacttc 60

tacatgtccg atgtttttgt gct 83

<210> SEQ ID NO 36
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P15_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 36

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnna ctcacacata 60

gtactgacac gtaagatagg atg 83

<210> SEQ ID NO 37
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P16_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 37

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnt taccctatct 60

cgtctatgta cgtcaggctg aat 83

<210> SEQ ID NO 38
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P17_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 38

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnna tcaacgtagg 60

gtaaggtcat atttttacct tac 83

<210> SEQ ID NO 39
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

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<223> OTHER INFORMATION: P18_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 39

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnt tccctctttc 60

tccgcttatg gatgaaagga cag 83

<210> SEQ ID NO 40
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P19_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 40

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnc ctgcacagtg 60

agtttctttc actctaactc tct 83

<210> SEQ ID NO 41
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P20_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 41

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnt gtcgctctag 60

tgtgactttt ccacctcgca tct 83

<210> SEQ ID NO 42
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P21_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 42

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnna tatctttctc 60

gggtaaagat taggcgtccg ata 83

<210> SEQ ID NO 43
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P23_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 43

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gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnc gattagccgt 60

agacgcaact cattgccgaa gat 83

<210> SEQ ID NO 44
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P24_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 44

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnt gtgagcattt 60

cagtacgagt gatgcagata aac 83

<210> SEQ ID NO 45
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P25_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 45

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnt atagttacca 60

agtactatgg gttggtggaa gcc 83

<210> SEQ ID NO 46
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P26_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 46

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnc caattatact 60

gtctgttatg ttctcggata agc 83

<210> SEQ ID NO 47
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P27_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 47

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnt caggtgcttg 60

taggctcatg ataggggtaa tgc 83

<210> SEQ ID NO 48

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<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P28_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 48

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnc tctgctgtaa 60

tctcagctcc acttgtttct aag 83

<210> SEQ ID NO 49
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P29_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 49

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnng tgcatattgc 60

agctgagcca gctcaatttg aag 83

<210> SEQ ID NO 50
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P30_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 50

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnc cgttgattta 60

cgcaacagcg gcttatatag ctc 83

<210> SEQ ID NO 51
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P31_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 51

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnc atcatcgaca 60

gttcgcagcc ctataacatg ata 83

<210> SEQ ID NO 52
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P32_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature

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<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 52

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnna tcgcaggatg 60

gtacagcatc atacatgatg agc 83

<210> SEQ ID NO 53
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P33_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 53

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnna tcgcaggatg 60

gtacagcatc atacatgatg agc 83

<210> SEQ ID NO 54
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P34_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 54

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnna tggcggtttc 60

gggtcctgca ctattcctaa taa 83

<210> SEQ ID NO 55
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P35_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 55

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnc cagtacgggt 60

actaataagt gtcatatcta ttg 83

<210> SEQ ID NO 56
<211> LENGTH: 83
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P36_PriExtUMI15_tpt
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(49)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 56

gtgactggag ttcagacgtg tgctcttccg atctnnnnnn nnnnnnnnnt gttggagagg 60

ttagaggtga ggaggcgaag ata 83

<210> SEQ ID NO 57
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 57

caagcagaag acggcatacg agatgtcggt aagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 58
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 58

caagcagaag acggcatacg agataggtca ctgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 59
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 59

caagcagaag acggcatacg agatgaatcc gagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 60
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 60

caagcagaag acggcatacg agatgtacct tggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 61
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 61

caagcagaag acggcatacg agatcatgag gagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 62
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

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<400> SEQUENCE: 62

caagcagaag acggcatacg agattgactg acgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 63
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 63

caagcagaag acggcatacg agatcgtatt cggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 64
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 64

caagcagaag acggcatacg agatctccta gagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 65
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 65

caagcagaag acggcatacg agattagttg cggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 66
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 66

caagcagaag acggcatacg agatgagata cggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 67
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 67

caagcagaag acggcatacg agataggtgt acgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 68

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<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 68

caagcagaag acggcatacg agattaatgc cggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 69
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 69

caagcagaag acggcatacg agattcagac gagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 70
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 70

caagcagaag acggcatacg agatgatagg ctgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 71
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 71

caagcagaag acggcatacg agattggtac aggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 72
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 72

caagcagaag acggcatacg agatcaaggt ctgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 73
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 73

caagcagaag acggcatacg agatgctatc ctgtgactgg agttcagacg tgtgctcttc 60

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cgatct 66

<210> SEQ ID NO 74
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 74

caagcagaag acggcatacg agatatggaa gggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 75
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 75

caagcagaag acggcatacg agattcaagg acgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 76
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 76

caagcagaag acggcatacg agatgttacg cagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 77
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 77

caagcagaag acggcatacg agatagtctg tggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 78
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 78

caagcagaag acggcatacg agatgcacgt aagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 79
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

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<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 79

caagcagaag acggcatacg agataacctt gggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 80
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 80

caagcagaag acggcatacg agatattgcg tggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 81
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 81

caagcagaag acggcatacg agatacctgg aagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 82
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 82

caagcagaag acggcatacg agatggagat gagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 83
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 83

caagcagaag acggcatacg agatgtactc tcgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 84
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 84

caagcagaag acggcatacg agatgtaacg acgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

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<210> SEQ ID NO 85
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 85

caagcagaag acggcatacg agatattcct ccgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 86
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 86

caagcagaag acggcatacg agatgtgttc ctgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 87
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 87

caagcagaag acggcatacg agataagcac tggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 88
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 88

caagcagaag acggcatacg agatctagca aggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 89
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 89

caagcagaag acggcatacg agattgcttc cagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 90
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 90

caagcagaag acggcatacg agatgcttag ctgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 91
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 91

caagcagaag acggcatacg agataaccgt tcgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 92
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 92

caagcagaag acggcatacg agatgacatt ccgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 93
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 93

caagcagaag acggcatacg agatagaccg tagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 94
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 94

caagcagaag acggcatacg agatgatact gggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 95
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 95

caagcagaag acggcatacg agattgcgta gagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 96
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 96

caagcagaag acggcatacg agattcggtt acgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 97
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 97

caagcagaag acggcatacg agatatgacg tcgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 98
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 98

caagcagaag acggcatacg agatgctgta aggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 99
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 99

caagcagaag acggcatacg agatgcaatg gagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 100
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 100

caagcagaag acggcatacg agatatctcg ctgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 101
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 101

caagcagaag acggcatacg agatggctat tggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 102
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 102

caagcagaag acggcatacg agatggtgtc ttgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 103
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 103

caagcagaag acggcatacg agattcaact gggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 104
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 104

caagcagaag acggcatacg agatcttcac cagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 105
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 105

caagcagaag acggcatacg agatacggtc ttgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 106
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 106

caagcagaag acggcatacg agattctcgc aagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 107
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 107

caagcagaag acggcatacg agatggaatt gcgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 108
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 108

caagcagaag acggcatacg agatacggat tcgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 109
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 109

caagcagaag acggcatacg agatttaagc gggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 110
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 110

caagcagaag acggcatacg agattgcagg tagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 111
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 111

caagcagaag acggcatacg agatcaatcg acgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 112
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 112

caagcagaag acggcatacg agatgtgcca tagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 113
<211> LENGTH: 66
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 113

caagcagaag acggcatacg agattgttcg aggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 114
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 114

caagcagaag acggcatacg agattggagt tggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 115
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 115

caagcagaag acggcatacg agatacgatg acgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 116
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 116

caagcagaag acggcatacg agattgatgt ccgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 117
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 117

caagcagaag acggcatacg agattgaacc tggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 118
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 118

caagcagaag acggcatacg agatcttcgt tcgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 119
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 119

caagcagaag acggcatacg agatcttctg aggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 120
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 120

caagcagaag acggcatacg agattgctca tggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 121
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 121

caagcagaag acggcatacg agatagttcg tcgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 122
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 122

caagcagaag acggcatacg agattagcgt ctgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 123
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 123

caagcagaag acggcatacg agatggcgtt atgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 124
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

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<400> SEQUENCE: 124

caagcagaag acggcatacg agatggtgat tcgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 125
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 125

caagcagaag acggcatacg agataacttg ccgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 126
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 126

caagcagaag acggcatacg agatgcaaga tcgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 127
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 127

caagcagaag acggcatacg agattcgcat tggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 128
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 128

caagcagaag acggcatacg agattgtaca ccgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 129
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 129

caagcagaag acggcatacg agatagctcc tagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 130
<211> LENGTH: 66

<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 130

caagcagaag acggcatacg agatgcaatt cggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 131
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 131

caagcagaag acggcatacg agatcttagg acgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 132
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 132

caagcagaag acggcatacg agatgtccta aggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 133
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 133

caagcagaag acggcatacg agataacgtg gagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 134
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 134

caagcagaag acggcatacg agatctgtgt tggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 135
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 135

caagcagaag acggcatacg agatgttaag gcgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 136
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 136

caagcagaag acggcatacg agatcacctt acgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 137
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 137

caagcagaag acggcatacg agattggtag ctgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 138
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 138

caagcagaag acggcatacg agatcagtga aggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 139
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 139

caagcagaag acggcatacg agatgttcaa ccgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 140
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 140

caagcagaag acggcatacg agattggcta tcgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 141
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

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<400> SEQUENCE: 141

caagcagaag acggcatacg agatctggag tagtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 142
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 142

caagcagaag acggcatacg agattctctt ccgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 143
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 143

caagcagaag acggcatacg agattctaac gcgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 144
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 144

caagcagaag acggcatacg agatggtcag atgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 145
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 145

caagcagaag acggcatacg agatctctgg ttgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 146
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 146

caagcagaag acggcatacg agattgtggt acgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 147

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<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 147

caagcagaag acggcatacg agatcctata ccgtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 148
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 148

caagcagaag acggcatacg agatttctct cggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 149
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 149

caagcagaag acggcatacg agatgtatgc tggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 150
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 150

caagcagaag acggcatacg agataagtcg aggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 151
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 151

caagcagaag acggcatacg agataaccga aggtgactgg agttcagacg tgtgctcttc 60

cgatct 66

<210> SEQ ID NO 152
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer

<400> SEQUENCE: 152

caagcagaag acggcatacg agattgttgt gggtgactgg agttcagacg tgtgctcttc 60

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cgatct 66

<210> SEQ ID NO 153
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P7 universal primer

<400> SEQUENCE: 153

caagcagaag acggcatacg a 21

<210> SEQ ID NO 154
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P5 universal primer

<400> SEQUENCE: 154

aatgatacgg cgaccaccga 20

<210> SEQ ID NO 155
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NEBNext i501 Primer

<400> SEQUENCE: 155

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cttccgatct 70

<210> SEQ ID NO 156
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NEBNext i502 Primer

<400> SEQUENCE: 156

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cttccgatct 70

<210> SEQ ID NO 157
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 157

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cttccgatct 70

<210> SEQ ID NO 158
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NEBNext i504 Primer

<400> SEQUENCE: 158

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cttccgatct 70

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<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: NEBNext i505 Primer

<400> SEQUENCE: 159

aatgatacgg cgaccaccga gatctacaca ggcgaagaca ctctttccct acacgacgct 60

cttccgatct 70

<210> SEQ ID NO 160
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: NEBNext i506 Primer

<400> SEQUENCE: 160

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cttccgatct 70

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NEBNext i507 Primer

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cttccgatct 70

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NEBNext i508 Primer

<400> SEQUENCE: 162

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cttccgatct 70

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<222> LOCATION: (65)..(78)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 163

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<221> NAME/KEY: misc_feature
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<223> OTHER INFORMATION: n is a, c, g, or t

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<223> OTHER INFORMATION: n is a, c, g, or t

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<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 167

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<223> OTHER INFORMATION: n is a, c, g, or t

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atctnnnnnn nnnnnnnnaa cggacaggat gcagcaaaat 100

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<223> OTHER INFORMATION: UMI14_i712
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<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 174

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atctnnnnnn nnnnnnnnaa cggacaggat gcagcaaaat 100

<210> SEQ ID NO 175
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<220> FEATURE:
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<223> OTHER INFORMATION: n is a, c, g, or t

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nnnnnnnnnn nnnntgcata ctggcaagat cggaagagca cacgtc 106

What is claimed is:

1. A method for spatially detecting at least one target analyte in a first location and a second location of a tissue sample comprising:

a) contacting the tissue sample with a plurality of nucleic acid probes, wherein each of the nucleic acid probes comprise a target binding domain that binds to the at least one target analyte, wherein the tissue sample [has been] is treated to facilitate binding of the nucleic acid probes to the target analyte;

b) collecting the nucleic acid probes, or portions thereof, bound to the at least one target analyte in a first location of the tissue sample under conditions that release the nucleic acid probes, or portions thereof, from the first location of the tissue sample;

c) collecting the nucleic acid probes, or portions thereof, bound to the at least one target analyte in a second location of the tissue sample under conditions that release the nucleic acid probes, or portions thereof, from the second location of the tissue sample;

d) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the first location of the tissue sample into each of the nucleic acid probes, or portions thereof, collected in step (b), thereby forming a first plurality of extension products that comprise the nucleic acid probes, or portions thereof, collected in step (b) and the at least one nucleic acid sequence that identifies the first location of the tissue sample;

e) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the second location of the tissue sample into each of the nucleic acid probes, or portions thereof, collected in step (c), thereby forming a second plurality extension

products that comprise the nucleic acid probes, or portions thereof, collected in step (c) and the at least one nucleic acid sequence that identifies the second location of the tissue sample; and

f) identifying the first plurality of extension products and the second plurality of extension products by sequencing the first plurality of extension products and the second plurality of extension products, thereby spatially detecting the at least one target analyte in the first location of the tissue sample and the second location of the tissue sample.

2. The method of claim **1**, wherein the tissue sample is a formalin-fixed paraffin-embedded (FFPE) tissue sample.

3. The method of claim **1**, wherein the tissue sample is immobilized onto a microscope slide.

4. The method of claim **3**, wherein the microscope slide comprises a plurality of primers immobilized on the microscope slide.

5. The method of claim **4**, wherein the plurality of primers is immobilized on the microscope slide at their 5' ends.

6. The method of claim **5**, wherein each of step (d) and step (e) comprise performing a solid-phase amplification reaction, wherein the solid-phase amplification reaction is carried out on the microscope slide using the plurality of primers immobilized on the microscope slide.

7. The method of claim **1**, wherein the sequencing step is performed using a next generation sequencing reaction.

8. The method of claim **1**, further comprising amplifying a library using the first plurality of extension products and the second plurality of extension products as templates.

9. The method of claim **1**, wherein the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one unique molecular identifier.

10. The method of claim **1**, wherein the at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one unique molecular identifier.

11. The method of claim **1**, wherein the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one amplification primer binding site.

12. The method of claim **1**, wherein the at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one amplification primer binding site.

13. The method of claim **1**, wherein the target binding domains comprise a single-stranded nucleic acid molecule.

14. The method of claim **1**, wherein steps (b) and (c) are performed simultaneously.

15. The method of claim **1**, wherein steps (d) and (e) are performed simultaneously.

16. A method for spatially detecting at least one target analyte in a first location and a second location of a tissue sample comprising:

a) contacting the tissue sample with a first plurality of nucleic acid probes and a second plurality of nucleic acid probes,

wherein each of the nucleic acid probes in the first plurality of nucleic acid probes comprise a target binding domain that binds to the at least one target analyte,

wherein each of the nucleic acid probes in the second plurality of nucleic acid probes comprise a target binding domain that binds to the at least one target analyte,

wherein the tissue sample is treated to facilitate binding of the first plurality of nucleic acid probes and the second plurality of nucleic acid probes to the at least one target analyte;

b) forming ligated probes by ligating each of nucleic acid probes from the first plurality of nucleic acid probes and each of nucleic acid probes from the second plurality of nucleic acid probes that are bound to each of identical molecules from the at least one target analyte;

c) collecting the ligated probes, or portions thereof, bound to each of the identical molecules from the at least one target analyte in a first location of the tissue sample under conditions that release the ligated probes, or portions thereof, from the first location of the tissue sample;

d) collecting the ligated probes, or portions thereof, bound to each of the identical molecules from the at least one target analyte in a second location of the tissue sample under conditions that release the probes, or portions thereof, from the second location of the tissue sample;

e) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the first location of the tissue sample into each of the ligated probes, or portions thereof, collected in step (c), thereby forming a first plurality of extension products that comprise the ligated probes, or portions thereof, collected in step (c) and the at least one nucleic acid sequence that identifies the first location of the tissue sample;

f) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the second location of the tissue sample into each of the ligated probes, or portions thereof, collected in step (d), thereby forming a second plurality extension products that comprise the ligated probes, or portions thereof, collected in step (d) and the at least one nucleic acid sequence that identifies the at least second location of the tissue sample; and

g) identifying the first plurality of extension products and the second plurality of extension products by sequencing the first plurality of extension products and the second plurality of extension products, thereby spatially detecting the at least one target analyte in the first location of the tissue sample and the second location of the tissue sample.

17. The method of claim **16**, wherein the tissue sample is a formalin-fixed paraffin-embedded (FFPE) tissue sample.

18. The method of claim **16**, wherein the tissue sample is immobilized onto a microscope slide.

19. The method of claim **18**, wherein the microscope slide comprises a plurality of primers immobilized on the microscope slide.

20. The method of claim **19**, wherein the plurality of primers is immobilized on the microscope slide at their 5' ends.

21. The method of claim **20**, wherein each of step (e) and step (f) comprise performing a solid-phase amplification reaction, wherein the solid-phase amplification reaction is carried out on the microscope slide using the plurality of primers immobilized on the microscope slide.

22. The method of claim **16**, wherein the sequencing step is performed using a next generation sequencing reaction.

23. The method of claim **16**, further comprising amplifying a library using the first plurality of extension products and the second plurality of extension products as templates.

24. The method of claim **16**, wherein the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one unique molecular identifier.

25. The method of claim **16**, wherein the at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one unique molecular identifier.

26. The method of claim **16**, wherein the nucleic acid probes in the first plurality of nucleic acid probes comprise an amplification primer binding site, the nucleic acid probes in the second plurality of nucleic acid probes comprise an amplification primer binding site, or both nucleic acid probes in the first plurality of nucleic acid probes comprise an amplification primer binding site and the nucleic acid probes in the second plurality of nucleic acid probes comprise an amplification primer binding site.

27. The method of claim **16**, wherein the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one amplification primer binding site, the at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one amplification primer binding site, or both the at least one nucleic acid sequence that identifies the first location of the tissue sample and the at least one nucleic acid sequence that identifies the second location of the tissue sample comprise at least one amplification primer binding site.

28. The method of claim **16**, wherein the target binding domain of each probe from the first plurality of nucleic acid probes and the second plurality of nucleic acid probes comprises a single-stranded nucleic acid molecule.

29. The method of claim **16**, wherein steps (c) and (d) are performed simultaneously.

30. The method of claim **16**, wherein steps (e) and (f) are performed simultaneously.

* * * * *

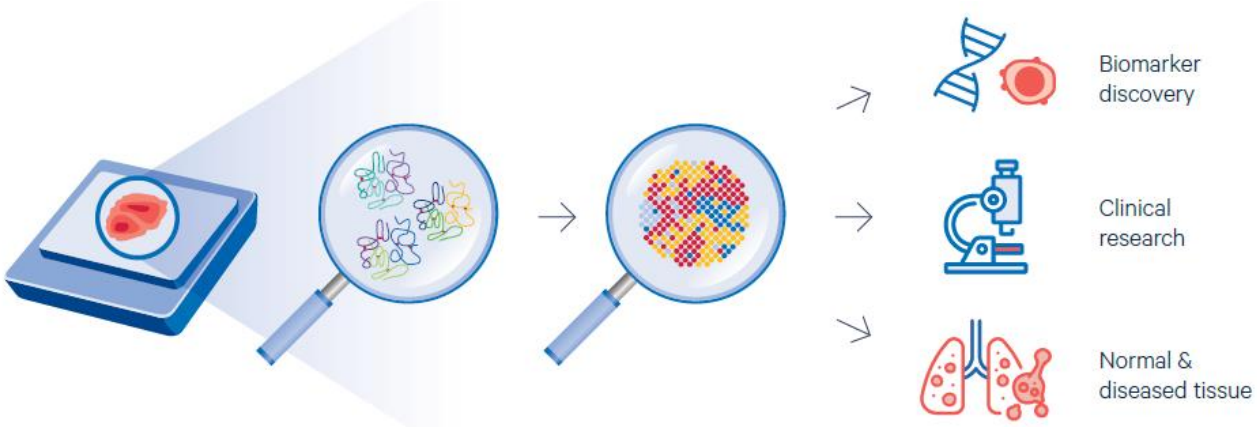
Exhibit 2

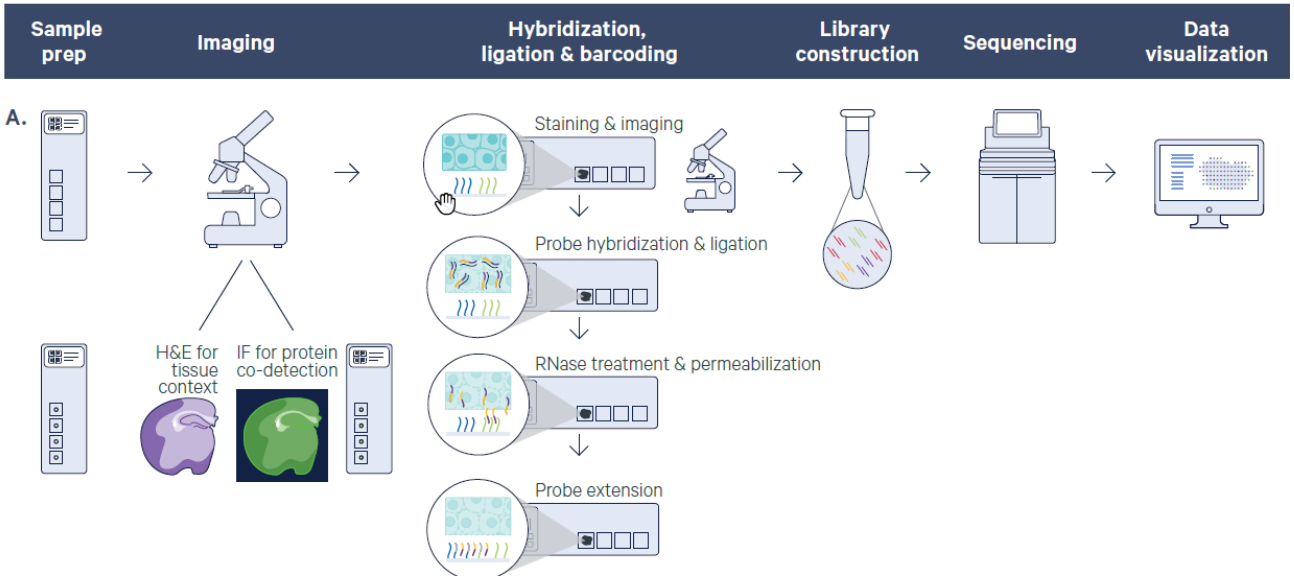
Exemplary Infringement of U.S. Patent No. 11,377,689 ("the '689 Patent")

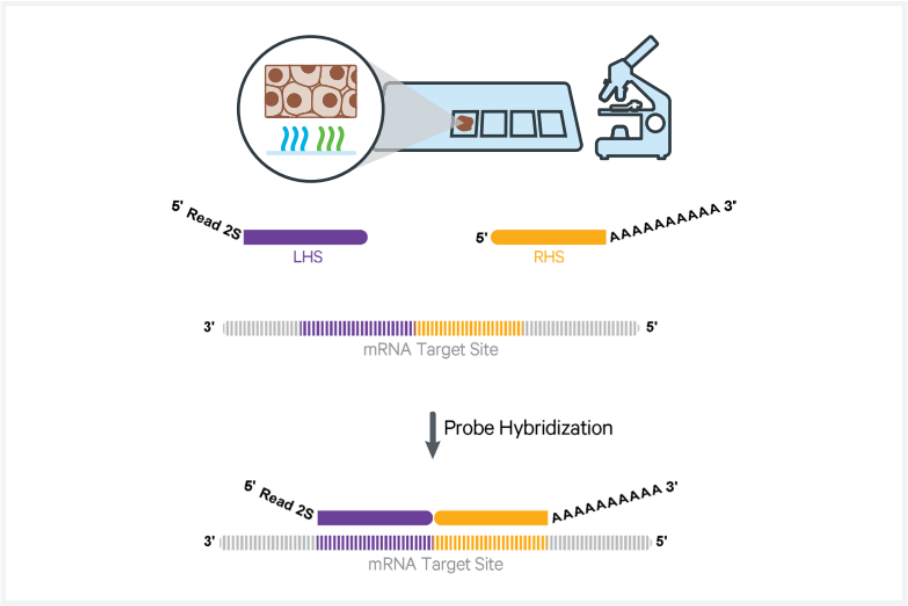
by use of 10X's Visium Spatial system ("Visium")

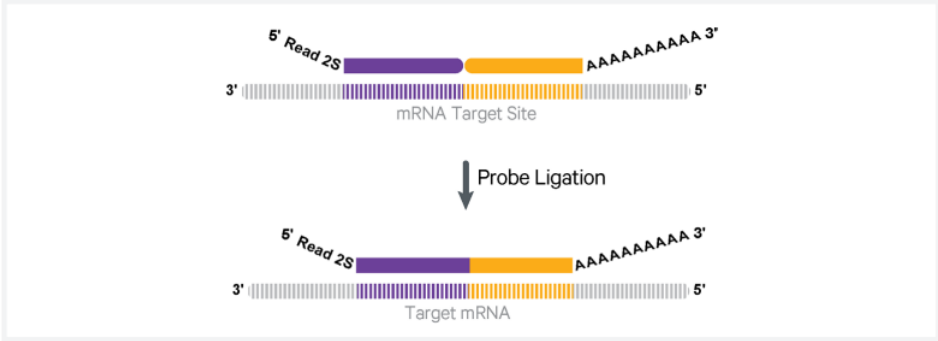
the '689 Patent	**10X's Visium Spatial System**
1. A method for spatially detecting at least one target analyte in a first location and a second location of a tissue sample comprising:	To the extent that the preamble is found to be limiting, 10X's Visium Spatial System ("Visium") and related products are designed and marketed such that they will be used, and are in fact used, to spatially detect at least one target analyte in a first location and a second location of a tissue sample. Visium and related products are all products, components, and services that are made, used, performed, offered to sell, sold, and/or imported in the United States by or on behalf of 10x in connection with 10x's Visium. Visium and related products include, for example and without limitation, Visium CytAssist, Visium Spatial Gene Expression slides, Visium Spatial Gene Expression reagents, and analysis and visualization software, Space Ranger and Loupe Browser, and Certified Service Providers (CSP), that are sold by 10X for use in 10X's Visium for spatially detecting target biological molecules via sample imaging and delivery of probes.[1]

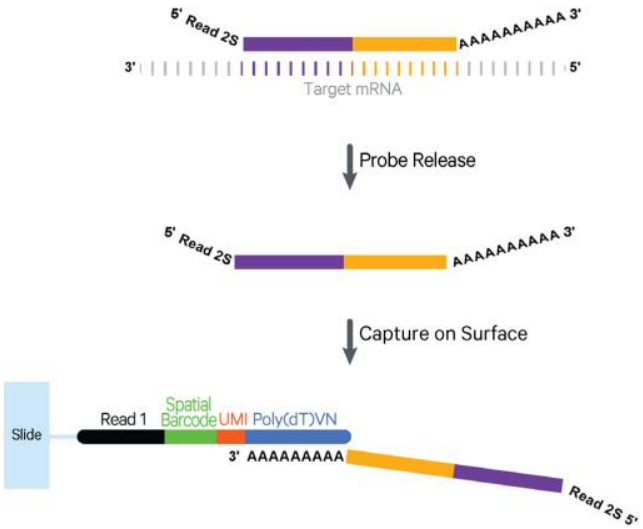
[1] Without limitation as to the named products, components, and services, these include but are not limited to: Dual Index Kit TS Set A (1000251); Dual Index Kit TT Set A (1000215); Human Gene Signature Panel (1000258); Human Immunology Panel (1000259); Human Neuroscience Panel (1000277); Human Pan-Cancer Panel (1000260); Library Amplification Kit (1000249); Target Hybridization Kit (1000248); Visium Accessory Kit (1000194); Visium CytAssist Calibration Kit (1000458); Visium CytAssist Reagent Accessory Kit (1000499); Visium CytAssist Spatial Gene Expression for FFPE, Mouse Transcriptome, 6.5mm (1000522); Visium CytAssist Spatial Gene Expression for FFPE, Mouse Transcriptome, 11mm (1000523); Visium CytAssist Spatial Gene Expression for FFPE, Human Transcriptome, 6.5mm (1000520); Visium CytAssist Spatial Gene Expression for FFPE, Human Transcriptome, 11mm (1000521); Visium CytAssist Training Kit (1000459); Visium Spatial for FFPE Gene Expression Kit, Human Transcriptome (1000338); Visium Spatial for FFPE Gene Expression Kit, Mouse Transcriptome (1000339); Visium Spatial for FFPE Gene Expression Starter Kit, Human Transcriptome (1000334); Visium Spatial for FFPE Gene Expression Starter Kit, Mouse Transcriptome (1000335); Visium Spatial Gene Expression Slide & Reagents Kit (1000187); Visium Spatial Gene Expression Starter Kit (1000200); Visium Spatial Tissue Optimization Slide & Reagents Kit (1000193); Visium Tissue Section Test Slides, 4 Slide Pack (1000347); Custom Panel Designer; Loupe Browser; Space Ranger; Certified Service Providers.

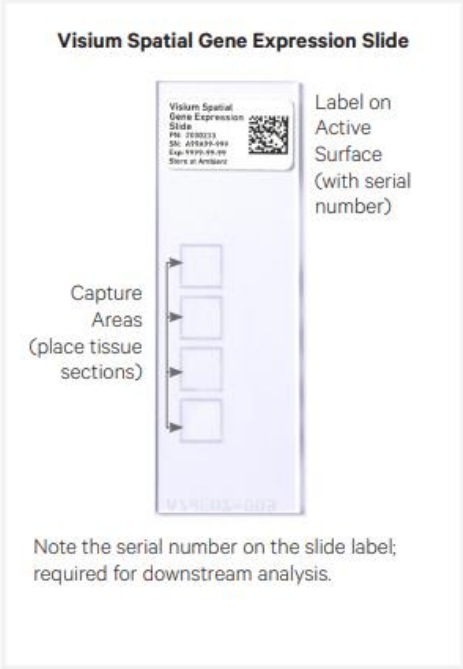
the ’689 Patent	10X’s Visium Spatial System
	“Visium Spatial Gene Expression for FFPE assays RNA levels by using probes against the whole transcriptome in intact formalin fixed paraffin embedded (FFPE) tissue sections and *maps the location(s) where gene activity is occurring*.” CG000407_VisiumSpatialGeneExpressionforFFPE_UserGuide_RevD at 18 (emphasis added). Visium Spatial Gene Expression for FFPE samples is a groundbreaking solution that complements traditional pathologist-led analysis. *Spatially profile RNA expression* for over 18,000 genes in human and mouse FFPE samples with high resolution across entire tissue sections. With whole transcriptome analysis and our specialized chemistry for FFPE tissue profiling, *you can detect any gene in any pathway, resolve tissue heterogeneity, and reveal the spatial organization of cell types and cell states within a morphological context*. Combine with immunofluorescence (IF) for simultaneous visualization of protein and gene expression.  10x_LIT000128_PS_Spatial_biology_without_limits_Spatial_gene_expression_in_FFPE at 1 (emphasis added).

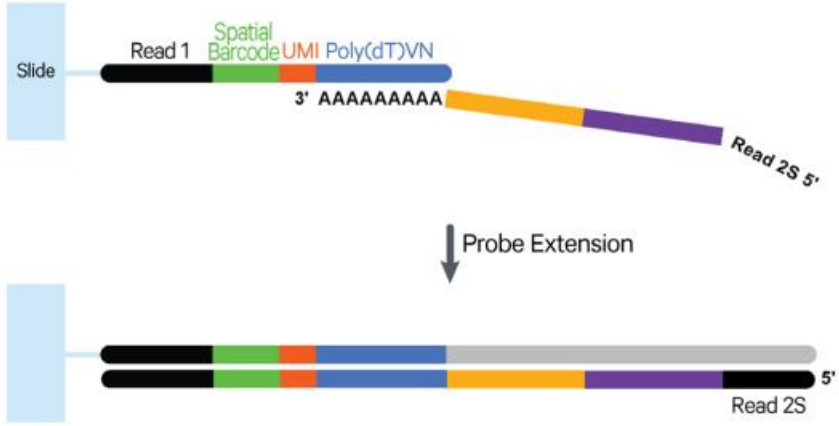
the ’689 Patent	10X’s Visium Spatial System
	An FFPE tissue section is placed onto a Visium gene expression slide and imaged for histological purposes (either H&E for morphological context or IF for protein co-detection). Each Capture Area on a Visium for FFPE slide has an array containing capture probes that bind to RNA. The probe pairs are extended to incorporate complements of the spatial barcodes, and sequencing libraries are prepared. The libraries are then sequenced and data visualized to determine *which genes are expressed, and where, as well as in what quantity*.  *Id.* at 2 (emphasis added). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.

the ’689 Patent	10X’s Visium Spatial System
a) contacting the tissue sample with a plurality of nucleic acid probes, wherein each of the nucleic acid probes comprise a target binding domain that binds to the at least one target analyte, wherein the tissue sample is treated to facilitate binding of the nucleic acid probes to the target analyte;	10X’s Visium performs contacting the tissue sample with a plurality of nucleic acid probes (probe pairs), wherein each of the nucleic acid probes comprise a target binding domain (LHS and RHS) that binds to the at least one target analyte, wherein the tissue sample is treated (deparaffinized, stained, and decrosslinked) to facilitate binding of the nucleic acid probes to the target analyte. “The human or mouse whole transcriptome probe panel, consisting of a *pair of specific probes* for each targeted gene, is added to the *deparaffinized, stained, and decrosslinked* tissues. Together, probe pairs hybridize to their complementary target RNA.” CG000407_VisiumSpatialGeneExpressionforFFPE_UserGuide_RevD at 19 (emphasis added).  “After hybridization, a ligase is added to seal the junction between the probe pairs that have hybridized to RNA, forming a ligation product.” *Id.*

the ’689 Patent	10X’s Visium Spatial System
	 To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
b) collecting the nucleic acid probes, or portions thereof, bound to the at least one target analyte in a first location of the tissue sample under conditions that release the nucleic acid probes, or portions thereof, from the first location of the tissue sample;	10X’s Visium performs collecting the nucleic acid probes, or portions thereof, bound to the at least one target analyte in a first location of the tissue sample under conditions that release the nucleic acid probes, or portions thereof, from the first location of the tissue sample. “The single stranded ligation products are released from the tissue upon RNase treatment and permeabilization, and then captured on the Visium slides.”

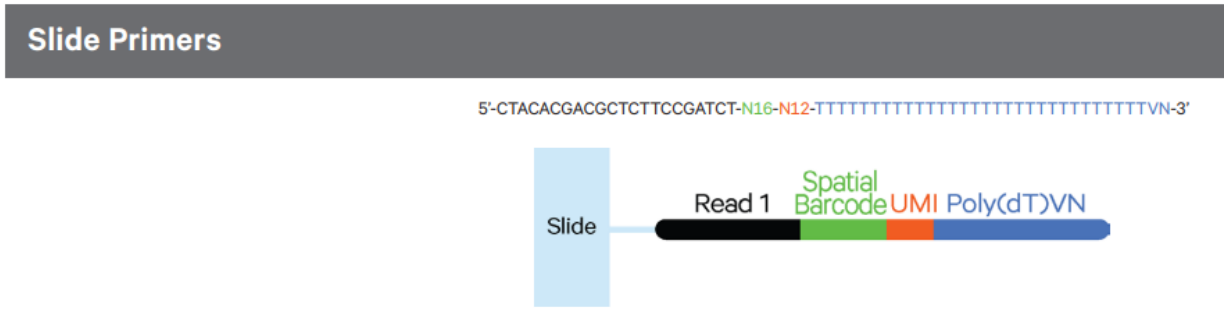
the ’689 Patent	10X’s Visium Spatial System
	 *Id.* at 20. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
c) collecting the nucleic acid probes, or portions thereof, bound to the at least one target analyte in a second location of the tissue sample under conditions that release the nucleic acid probes, or portions thereof, from the	10X’s Visium performs collecting the nucleic acid probes, or portions thereof, bound to the at least one target analyte in a second location of the tissue sample under conditions that release the nucleic acid probes, or portions thereof, from the second location of the tissue sample. Reagents are added to and reactions are performed on at least 5000 distinct locations (gene expression spots) simultaneously: “Always cover the tissue section completely when adding reagents to the well. A gentle tap may help spread the reagent more evenly.” *Id.* at 29.

the ’689 Patent	10X’s Visium Spatial System
second location of the tissue sample;	“Visium slides include 4 Capture Areas (6.5 x 6.5 mm), each with ~5,000 unique gene expression spots. Each gene expression spot includes primers with a unique Spatial Barcode . . . . The tissue sections are always placed on the active surface of the Capture Areas.”  *Id.* at 23. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
d) performing an extension reaction that incorporates at least one nucleic acid	10X’s Visium performs an extension reaction that incorporates at least one nucleic acid sequence (primer sequence containing spatial barcode) that identifies the first location of the tissue sample into each of the nucleic acid probes, or portions thereof, collected in step (b), thereby forming a first

the '689 Patent	10X's Visium Spatial System
sequence that identifies the first location of the tissue sample into each of the nucleic acid probes, or portions thereof, collected in step (b), thereby forming a first plurality of extension products that comprise the nucleic acid probes, or portions thereof, collected in step (b) and the at least one nucleic acid sequence that identifies the first location of the tissue sample;	plurality of extension products (spatially barcoded, ligated probe products) that comprise the nucleic acid probes, or portions thereof, collected in step (b) and the at least one nucleic acid sequence (spatial barcode) that identifies the first location of the tissue sample. For example: "Once ligation products are captured, probes are extended by the addition of UMI, Spatial Barcode and partial Read 1. This generates spatially barcoded, ligated probe products, which can then be carried forward for library preparation."  *Id.* at 20. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the '689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
e) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the second location of the	*See Supra* infringement analysis for Claims 1(c), 1(d). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the '689 Patent, infringement of such limitations can be found under the doctrine of equivalents.

the ’689 Patent	10X’s Visium Spatial System
tissue sample into each of the nucleic acid probes, or portions thereof, collected in step (c), thereby forming a second plurality extension products that comprise the nucleic acid probes, or portions thereof, collected in step (c) and the at least one nucleic acid sequence that identifies the second location of the tissue sample; and	
f) identifying the first plurality of extension products and the second plurality of extension products by sequencing the first plurality of extension products and the second plurality of extension products, thereby spatially detecting the at least one target analyte in the first location of the tissue smaple and the second location of the tissue sample.	10X’s Visium performs identifying the first plurality of extension products and the second plurality of extension products by sequencing the first plurality of extension products and the second plurality of extension products, thereby spatially detecting the at least one target analyte in the first location of the tissue smaple and the second location of the tissue sample. *See Supra* infringement analysis for Claim 1(c). Sequence of the extension products is determined by the application of Illumina-specific adapters followed by amplification for assessment by a sequencer: “TruSeq Read 1 and Small RNA Read 2 (Read 2S) are standard Illumina sequencing primer sites used in paired-end sequencing. TruSeq Read 1 is used to sequence 16 bp Spatial Barcode and 12 bp UMI. Small RNA Read 2 (Read 2S) is used to sequence the Ligated Probe Insert.” *Id.* at 60. 

the '689 Patent	**10X's Visium Spatial System**
	To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the '689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
2. The method of claim 1, wherein the tissue sample is a formalin-fixed paraffin-embedded (FFPE) tissue sample.	10X's Visium includes a formalin-fixed paraffin-embedded (FFPE) tissue sample. "Visium Spatial Gene Expression for FFPE assays RNA levels by using probes against the whole transcriptome in intact formalin fixed paraffin embedded (FFPE) tissue sections and maps the location(s) where gene activity is occurring." *Id.* at 18. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the '689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
3. The method of claim 1, wherein the tissue sample is immobilized onto a microscope slide.	10X's Visium includes a tissue sample that is immobilized onto a microscope slide. "Tissue sections placed on these Capture Areas are *fixed* and stained . . . ." CG000239_Visium_Spatial_Gene_Expression_User_Guide_Rev_F at 16 (emphasis added). "Immediately place a finger on the backside of the Capture Area on the slide for a few seconds to *allow the section to adhere to the slide*." TissuePreparationGuide at 15 (emphasis added). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the '689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
4. The method of claim 6, wherein the microscope slide comprises a plurality of primers immobilized on the microscope slide.	10X's Visium includes a microscope slide comprises a plurality of primers immobilized on the microscope slide. "Tissue sections are placed within the frames of Capture Areas on Visium Spatial slides. Only one section should be placed within each Capture Area." *Id.* at 10.

the ’689 Patent	10X’s Visium Spatial System
	“Each Visium Spatial or Gateway Gene Expression Slide contains Capture Areas with gene expression spots that include primers required for capture and priming of poly-adenylated mRNA.” CG000239_Visium_Spatial_Gene_Expression_User_Guide_Rev_F at 16. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
5. The method of claim 7, wherein the plurality of primers is immobilized on the microscope slide at their 5' ends.	10X’s Visium includes plurality of primers immobilized on the microscope slide at their 5' ends.  CG000407_VisiumSpatialGeneExpressionforFFPE_UserGuide_RevD at 71. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
6. The method of claim 8, wherein each of step (d) and step (e) comprise performing a solid-phase amplification reaction, wherein the solid-phase	*See supra* infringement analysis for Claims 1(c), 1(d), 1(e). “Seq-Scope is based on a solid-phase amplification of randomly barcoded single-molecule oligonucleotides using an Illumina sequencing platform.” https://www.10xgenomics.com/resources/publications?query=34115981&pmid=34115981

the ’689 Patent	10X’s Visium Spatial System
amplification reaction is carried out on the microscope slide using the plurality of primers immobilized on the microscope slide.	
7. The method of claim 1, wherein the sequencing step is performed using a next generation sequencing reaction.	10X’s Visium performs the sequencing step using a next generation sequencing reaction. “The resulting 10x barcoded library is compatible with standard NGS short-read sequencing on Illumina sequencers for massive transcriptional profiling of entire tissue sections.” https://www.10xgenomics.com/products/spatial-gene-expression. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
8. The method of claim 1, further comprising amplifying a library using the first plurality of extension products and the second plurality of extension products as templates.	10X’s Visium performs amplifying a library using the first plurality of extension products and the second plurality of extension products as templates. “The spatially barcoded, ligated probe products are released from the slide and harvested for qPCR to determine Sample Index PCR cycle number. The products then undergo indexing via Sample Index PCR. This, in turn, generates final library molecules that are cleaned up by SPRIselect, assessed on a bioanalyzer or a similar instrument, quantified, and then sequenced.” CG000407_VisiumSpatialGeneExpressionforFFPE_UserGuide_RevD at 21. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
9. The method of claim 1, wherein the at least one nucleic acid sequence that identifies the first location	10X’s Visium includes at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one unique molecular identifier.

the ’689 Patent	**10X’s Visium Spatial System**
of the tissue sample comprises at least one unique molecular identifier.	“[E]ach spot with primers that include: Illumina TruSeq Read 1 (partial read 1 sequencing primer); 16 nt Spatial Barcode (all primers in a specific spot share the same Spatial Barcode); 12 nt unique molecular identifier (UMI); 30 nt poly(dT) sequence (captures ligation product).” *Id.* at 18. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
10. The method of claim 1, wherein the at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one unique molecular identifier.	*See supra* infringement analysis for Claims 1(c), 12. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
11. The method of claim 1, wherein the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one amplification primer binding site.	10X’s Visium includes the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one amplification primer binding site. “TruSeq Read 1 and Small RNA Read 2 (Read 2S) are standard Illumina sequencing primer sites used in paired-end sequencing.”

the ’689 Patent	10X’s Visium Spatial System
	 CG000407_VisiumSpatialGeneExpressionforFFPE_UserGuide_RevD at 21. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
12. The method of claim 1, wherein the at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one amplification primer binding site.	*See supra* infringement analysis for Claims 1(c), 15. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
13. The method of claim 1, wherein the target binding domains comprise a single-stranded nucleic acid molecule.	10X’s Visium includes target binding domains comprise a single-stranded nucleic acid molecule. *See supra* infringement analysis for Claim 1(a). “The single stranded ligation products are released from the tissue upon RNase treatment and permeabilization, and then captured on the Visium slides.” *Id.* at 20.

the ’689 Patent	**10X’s Visium Spatial System**
	To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
14. The method of claim 1, wherein steps (b) and (c) are performed simultaneously.	10X’s Visium performs steps (b) and (c) of claim 1 simultaneously. *See supra* infringement analysis for Claim 1(b), 1(c). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
15. The method of claim 1, wherein steps (d) and (e) are performed simultaneously.	10X’s Visium performs steps (d) and (e) of claim 1 simultaneously. *See supra* infringement analysis for Claims 1(c), 1(d), 1(e). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
16. A method for spatially detecting at least one target analyte in a first location and a second location of a tissue sample comprising:	To the extent that the preamble is found to be limiting, 10X’s Visium, including instruments and consumables, is designed and marketed such that it will be used, and is in fact used, to spatially detect at least one target analyte in a first location and a second location of a tissue sample. *See supra* infringement analysis for Claim 1. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
a) contacting the tissue sample with a first plurality of nucleic acid	10X’s Visium performs contacting the tissue sample with a first plurality of nucleic acid probes (LHS probes) and a second plurality of nucleic acid probes (RHS probes).

the ’689 Patent	10X’s Visium Spatial System
probes and a second plurality of nucleic acid probes,	*See supra* infringement analysis for Claim 1(a). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
wherein each of the nucleic acid probes in the first plurality of nucleic acid probes comprise a target binding domain that binds to the at least one target analyte,	10X’s Visium includes nucleic acid probes in the first plurality of nucleic acid probes comprise a target binding domain (LHS target specific sequence) that binds to the at least one target analyte. *See supra* infringement analysis for Claim 1(a). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
wherein each of the nucleic acid probes in the second plurality of nucleic acid probes comprise a target binding domain that binds to the at least one target analyte,	10X’s Visium includes nucleic acid probes in the second plurality of nucleic acid probes comprise a target binding domain (RHS target specific sequence) that binds to the at least one target analyte. *See supra* infringement analysis for Claim 1(a). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
wherein the tissue sample is treated to facilitate binding of the first plurality of nucleic acid probes and the second plurality of nucleic acid probes to the at least one target analyte;	10X’s Visium treats tissue sample to facilitate binding of the first plurality of nucleic acid probes and the second plurality of nucleic acid probes to the at least one target analyte. “FFPE tissue sections should be deparaffinized, stained, imaged, and decrosslinked before proceeding with Visium Spatial Gene Expression for FFPE.” CG000407_VisiumSpatialGeneExpressionforFFPE_UserGuide RevD at 37. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.

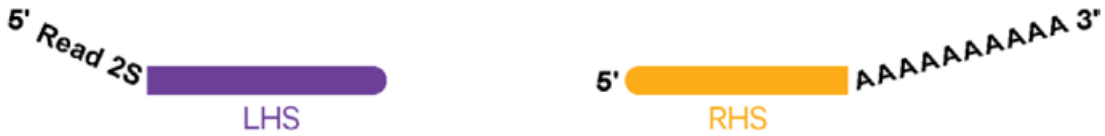
the ’689 Patent	10X’s Visium Spatial System
b) forming ligated probes by ligating each of nucleic acid probes from the first plurality of nucleic acid probes and each of nucleic acid probes from the second plurality of nucleic acid probes that are bound to each of identical molecules from the at least one target analyte;	10X’s Visium performs forming ligated probes (ligation products) by ligating each of nucleic acid probes from the first plurality of nucleic acid probes and each of nucleic acid probes from the second plurality of nucleic acid probes that are bound to each of identical molecules from the at least one target analyte. *See supra* infringement analysis for Claim 1(a). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
c) collecting the ligated probes, or portions thereof, bound to the at least one target analyte in a first location of the tissue sample under conditions that release the ligated probes, or portions thereof, from the first location of the tissue sample;	10X’s Visium performs collecting the ligated probes, or portions thereof, bound to the at least one target analyte in a first location of the tissue sample under conditions that release the ligated probes, or portions thereof, from the first location of the tissue sample. *See supra* infringement analysis for Claim 1(b). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
d) collecting the ligated probes, or portions thereof, bound to each of identical molecules from the at least one target analyte in a second location of the tissue sample under conditions that release the probes, or portions thereof,	10X’s Visium performs collecting the ligated probes, or portions thereof, bound to each of identical molecules from the at least one target analyte in a second location of the tissue sample under conditions that release the probes, or portions thereof, from the second location of the tissue sample. *See supra* infringement analysis for Claims 1(c), 20(c). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.

the ’689 Patent	**10X’s Visium Spatial System**
from the second location of the tissue sample;	
e) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the first location of the tissue sample into each of the ligated probes, or portions thereof, collected in step (c), thereby forming a first plurality of extension products that comprise the ligated probes, or portions thereof, collected in step (c) and the at least one nucleic acid sequence that identifies the first location of the tissue sample;	10X’s Visium performs performing an extension reaction that incorporates at least one nucleic acid sequence (primer sequence containing spatial barcode) that identifies the first location of the tissue sample into each of the ligated probes, or portions thereof, collected in step (c), thereby forming a first plurality of extension products (spatially barcoded, ligated probe products) that comprise the ligated probes, or portions thereof, collected in step (c) and the at least one nucleic acid sequence that identifies the first location of the tissue sample. *See supra* infringement analysis for Claim 1(d). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
f) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the second location of the tissue sample into each of the ligated probes, or portions thereof, collected in step (d), thereby forming a second plurality extension products that comprise the ligated	10X’s Visium performs an extension reaction that incorporates at least one nucleic acid sequence that identifies the second location of the tissue sample into each of the ligated probes, or portions thereof, collected in step (d), thereby forming a second plurality extension products that comprise the ligated probes, or portions thereof, collected in step (d) and the at least one nucleic acid sequence that identifies the at least second location of the tissue sample. *See supra* infringement analysis for Claims 1(c), 20(f). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.

the ’689 Patent	10X’s Visium Spatial System
probes, or portions thereof, collected in step (d) and the at least one nucleic acid sequence that identifies the at least second location of the tissue sample; and	
g) identifying the first plurality of extension products and the second plurality of extension products by sequencing the first plurality of extension products and the second plurality of extension products, thereby spatially detecting the at least one target analyte in the first location and the second location of the tissue sample.	10X’s Visium performs identifying the first plurality of extension products and the second plurality of extension products by sequencing the first plurality of extension products and the second plurality of extension products, thereby spatially detecting the at least one target analyte in the first location and the second location of the tissue sample. *See supra* infringement analysis for Claim 1(f). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
17. The method of claim 16, wherein the tissue sample is a formalin-fixed paraffin-embedded (FFPE) tissue sample.	10X’s Visium includes a formalin-fixed paraffin-embedded (FFPE) tissue sample. *See supra* infringement analysis for Claim 4. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
18. The method of claim 16, wherein the tissue sample is immobilized onto a microscope slide.	10X’s Visium includes tissue sample immobilized onto a microscope slide. *See supra* infringement analysis for Claim 6.

the ’689 Patent	10X’s Visium Spatial System
	To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
19. The method of claim 18, wherein the microscope slide comprises a plurality of primers immobilized on the microscope slide.	10X’s Visium includes a microscope slide comprises a plurality of primers immobilized on the microscope slide. *See supra* infringement analysis for Claim 7. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
20. The method of claim 19, wherein the plurality of primers is immobilized on the microscope slide at their 5' ends.	10X’s Visium includes a plurality of primers is immobilized on the microscope slide at their 5' ends. *See supra* infringement analysis for Claim 8. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
21. The method of claim 20, wherein each of step (e) and step (f) comprise performing a solid-phase amplification reaction, wherein the solid-phase amplification reaction is carried out on the microscope slide using the plurality of primers immobilized on the microscope slide.	10X’s Visium performs the method of claim 24, wherein each of step (e) and step (f) comprise performing a solid-phase amplification reaction, wherein the solid-phase amplification reaction is carried out on the microscope slide using the plurality of primers immobilized on the microscope slide. *See supra* infringement analysis for Claim 9. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
22. The method of claim 16, wherein the sequencing	10X’s Visium performs sequencing using a next generation sequencing reaction.

the ’689 Patent	10X’s Visium Spatial System
step is performed using a next generation sequencing reaction.	*See supra* infringement analysis for Claim 10. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
23. The method of claim 16, further comprising amplifying a library using the first plurality of extension products and the second plurality of extension products as templates.	10X’s Visium performs amplifying a library using the first plurality of extension products and the second plurality of extension products as templates. *See supra* infringement analysis for Claim 11. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
24. The method of claim 16, wherein the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one unique molecular identifier.	10X’s Visium includes the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one unique molecular identifier. *See supra* infringement analysis for Claim 12. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
25. The method of claim 16, wherein the at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one unique molecular identifier.	10X’s Visium includes the at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one unique molecular identifier. *See supra* infringement analysis for Claims 1(c), 28. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
26. The method of claim 16, wherein the nucleic	10X’s Visium includes nucleic acid probes in the first plurality of nucleic acid probes comprise an amplification primer binding site (Read 2), the nucleic acid probes in the second plurality of nucleic

the ’689 Patent	10X’s Visium Spatial System
acid probes in the first plurality of nucleic acid probes comprise an amplification primer binding site, the nucleic acid probes in the second plurality of nucleic acid probes comprise an amplification primer binding site, or both nucleic acid probes in the first plurality of nucleic acid probes comprise an amplification primer binding site and the nucleic acid probes in the second plurality of nucleic acid probes comprise an amplification primer binding site.	acid probes comprise an amplification primer binding site, or both nucleic acid probes in the first plurality of nucleic acid probes comprise an amplification primer binding site and the nucleic acid probes in the second plurality of nucleic acid probes comprise an amplification primer binding site. “TruSeq Read 1 and Small RNA Read 2 (Read 2S) are standard Illumina sequencing primer sites used in paired-end sequencing.” CG000407_VisiumSpatialGeneExpressionforFFPE_UserGuide_RevD at 21.  *Id.* at 19. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
27. The method of claim 16, wherein the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one amplification primer binding site, the at least one nucleic acid sequence that identifies the second location of the tissue	10X’s Visium includes the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one amplification primer binding site, the at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one amplification primer binding site, or both the at least one nucleic acid sequence that identifies the first location of the tissue sample and the at least one nucleic acid sequence that identifies the second location of the tissue sample comprise at least one amplification primer binding site. *See supra* infringement analysis for Claim 15. To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.

the ’689 Patent	10X’s Visium Spatial System
sample comprises at least one amplification primer binding site, or both the at least one nucleic acid sequence that identifies the first location of the tissue sample and the at least one nucleic acid sequence that identifies the second location of the tissue sample comprise at least one amplification primer binding site.	
28. The method of claim 16, wherein the target binding domain of each probe from the first plurality of nucleic acid probes and the second plurality of nucleic acid probes comprises a single-stranded nucleic acid molecule.	10X’s Visium includes target binding domain of each probe from the first plurality of nucleic acid probes and the second plurality of nucleic acid probes comprises a single-stranded nucleic acid molecule. *See supra* infringement analysis for Claims 17, 20(a). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
29. The method of claim 16, wherein steps (c) and (d) are performed simultaneously.	10X’s Visium performs steps (c) and (d) of claim 20 simultaneously. *See supra* infringement analysis for Claims 1(c), 20(c), 20(d). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.
30. The method of claim 16, wherein steps (e) and	10X’s Visium performs steps (e) and (f) of claim 20 simultaneously.

the ’689 Patent	**10X’s Visium Spatial System**
(f) are performed simultaneously.	*See supra* infringement analysis for Claims 1(c), 20(e), 20(f). To the extent Visium and related products are argued by Counterclaim-Defendant or found by the Court not to literally infringe certain limitations of the ’689 Patent, infringement of such limitations can be found under the doctrine of equivalents.